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Chris Galanos

T H E S E

présentée

A L A F A C U L T E D E S S C I E N C E S D ' O R S A Y
U N I V E R S I T E D E P A R I S

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(mention sciences)

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Chris Galanos

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Sujet de la thèse:

Activité biologique de lipopolysaccharides et du lipide A.

Soutenue le

devant la Commission d'Examen

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ABBREVIATIONS

LPS	Lipopolysaccharide
ET	Endotoxin
S form	Smooth form
R form	Rough form
KDO	2-Keto-3-deoxyoctonate
Glc	Glucose
Gal	Galactose
Hep	Heptose
GlcNAc	N-acetyl glucosamine
GalNAc	N-acetyl galactosamine
PEN	Phosphoethanolamine
P-PEN	Pyrophosphoethanolaminé
BSA	Bovine serum albumine
C	Complement
SRC	Sheep red cells
HRC	Human red cells
NaD	Sodium deoxycholate
LD50	Lethal dosis 50%
ED50	Effective dosis 50%
MPD-2	Minimum pyrogenic dosis 3 hours after injection
BCG	Bacillus Calmette-Guérin
n. d.	not determined

Je tiens à exprimer ma plus profonde gratitude à Monsieur le Professeur E. Lederer pour l'honneur qu'il me fait en acceptant d'être le Président du jury de cette thèse ainsi que pour les conseils et suggestions qu'il a apporté à sa préparation.

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Resumé

Ce résumé est un condensé du travail réalisé par l'auteur lors des dernières années dans le domaine des lipopolysaccharides. Une partie des résultats présentés dans le chapitre "Endotoxic activities of lipid A" furent obtenus lors d'un stage dans le laboratoire du Dr. Chedid à l'Institut Pasteur de Paris. Le travail se rapporte tant au développement de méthodes concernant l'isolement et la purification de ces macromolécules qu'à leurs propriétés biologiques. Une attention particulière a été accordée à la partie de la molécule responsable de l'activité biologique et de ses propriétés immunogènes.

Les lipopolysaccharides sont constitués par le lipide A, le polysaccharide du noyau ou "Core" et le polysaccharide O-spécifique. Le polysaccharide O-spécifique représente la partie variable de la molécule. La structure du polysaccharide du core est très semblable chez les différentes Entérobactéries et identique au sein d'un même genre. Le lipide A est un constituant commun pour les bactéries à gram négatif. Sa structure est similaire sinon identique chez les Entérobactéries.

Les mutants rugueux ou "rough" des bactéries à gram négatif synthétisent des lipopolysaccharides défectueux, dépourvus de polysaccharides O-spécifiques et dont le core de base est plus ou moins complet.

La structure chimique des lipopolysaccharides a pu être élucidée grâce à l'étude de la structure de fragments obtenus après hydrolyse partielle. Le faible rendement en oligosaccharides a toujours été un problème majeur dans ce domaine. Une méthode d'hydrolyse basée sur le principe d'une hydrolyse graduelle réalisée en continu et couplée à une dialyse est décrite dans cette thèse (Publication No. I). La méthode est très simple, car on peut faire varier les différents facteurs influençant l'hydrolyse indépendamment les uns des autres et ainsi améliorer le rendement en oligosaccharides.

Durant les 10 dernières années l'isolement des mutants "rough" a ouvert de nouveaux horizons dans le domaine de la recherche sur les lipopolysaccharides. Leurs lipopolysaccharides se sont avérés d'excellents matériaux de départ pour des études chimiques et pour la biosynthèse de la partie interne de la molécule.

Pourtant leur isolement par les diverses méthodes existantes est souvent loin d'être satisfaisant. Grâce à la méthode décrite dans la publication No. II les lipopolysaccharides "rough" (R) ont pu être extraits spécifiquement. Les bactéries sèches sont traitées par un mélange monophasique de phénol-chloroforme-éther de pétrole à basse température. On obtient avec un très bon rendement des préparations solubles dans l'eau et dépourvues d'acides nucléiques et de contaminants protéiques.

Les acides nucléiques et les protéines mis à part, les lipopolysaccharides contiennent toujours additionnellement des substances à faible poids moléculaire tels que les cations Mg^{++} et Ca^{++} et des amines basiques telles que la putrescine, la spermidine, la spermine et l'éthanolamine. Il a toujours été difficile de les éliminer des lipopolysaccharides.

Récemment il a été possible de les séparer des lipopolysaccharides grâce à un procédé basé sur l'électrodialyse (publication III). Les résultats obtenus suggèrent que ces substances basiques sont présentes dans la molécule sous forme de gels avec des groupements acides. On a pu montrer en outre que tous les lipopolysaccharides d'Entérobactéries (S ou R) sont fondamentalement des substances acides; ceci est dû à la présence prédominante de groupements acides. Pourtant les lipopolysaccharides isolés à partir des bactéries par diverses méthodes, semblent toujours être neutres. Ceci est dû au fait qu'ils sont toujours extraits en même temps que les cations métalliques et les amines basiques citées plus haut. Les deux groupes de substances basiques, cations métalliques et amines basiques ont des effets opposés sur les propriétés physiques des lipopolysaccharides. Ainsi Mg^{++} et Ca^{++} , d'une part,

favorisent la formation de gros agrégats qui provoquent une diminution de la solubilité. Les amines basiques, d'autre part, ont un effet désagrégeant qui entraîne une augmentation de la solubilité. C'est pourquoi dans les lipopolysaccharides extraits à partir de bactéries, des variations fortuites de la concentration en cations métalliques et en amines basiques donnent lieu à des produits ayant des propriétés physiques différentes. L'électrodialyse des lipopolysaccharides, suivie de la neutralisation par les alcalis ou une amine basique particulière, permet d'obtenir des préparations extrêmement solubles et dont la vitesse de sédimentation à l'ultracentrifugation est grandement réduite. L'électrodialyse mène à des préparations mieux définies qui pourraient être utiles pour des études biologiques et servir de matériaux de départ dans des questions concernant la structure des membranes et des parois.

Activité biologique

Les lipopolysaccharides sont des substances biologiquement actives et responsables d'un bon nombre des activités endotoxiques. Ils peuvent sans addition supplémentaire d'anticorps réagir in vitro avec le complément et provoquer la perte de son activité hémolytique.

L'interaction entre les lipopolysaccharides et le complément de cobaye, la région de la molécule lipopolysaccharidique responsable de cette interaction et le degré de participation des anticorps naturels ont été étudiés dans cette thèse (Publications No. IV, V).

L'activité anti-complémentaire est obtenue uniquement avec des lipopolysaccharides solubles. Cette activité est négligeable dans le cas de préparations insolubles mais elle devient très élevée quand on ajoute l'anticorps spécifique correspondant. L'étude du rôle des cations divalents Mg^{++} et Ca^{++} a montré que ceux-ci précipitent les lipopolysaccharides R, provoquant ainsi une perte de l'activité anti-complémentaire. Par contre les lipopolysaccharides des formes lisses ou smooth (S), sont moins sensibles à l'action précipitante de ces ions et leur activité reste en principe inchangée. Si la solubilité

est un facteur nécessaire à l'activité anti-complémentaire ce n'est pourtant pas un facteur suffisant et il faut remarquer que tous les lipopolysaccharides solubles ne présentent pas cette activité.

L'étude d'un grand nombre de préparations solubles de lipopolysaccharides de formes S ou R, obtenues à partir de Salmonella, Arizona et Escherichia coli a montré qu'un tiers environ était hautement actif, un tiers moyennement actif et le reste pratiquement inactif.

Ces différences dans l'activité anti-complémentaire sont à la fois qualitatives et quantitatives. Pourtant elles ne dépendent ni du genre des bactéries dont proviennent les lipopolysaccharides ni de la classe R ou S à laquelle ces bactéries appartiennent. En d'autres termes, qu'un lipopolysaccharide réagisse avec le complément ou non est indépendant de la qualité et de la quantité de sucres présents dans la molécule. Les lipopolysaccharides utilisés lors de ces études sont des préparations hautement endotoxiques; on peut en déduire que la toxicité d'un lipopolysaccharide et son activité anti-complémentaire in vitro ne sont pas toujours parallèles.

Le lipide A représente la partie de la molécule responsable de l'activité anti-complémentaire.

Le lipide A libre, obtenu par hydrolyse acide du lipopolysaccharide, est totalement insoluble et ne montre aucune activité anti-complémentaire. La solubilisation du lipide A a été obtenue en le complexant avec l'albumine du sérum de boeuf (BSA). Un grand nombre de complexes lipide A/BSA ainsi préparés furent testés pour leur activité anti-complémentaire.

Toutes les préparations se montrèrent hautement et comparablement actives. Cette activité est indépendante du lipopolysaccharide d'origine à partir duquel les préparations de lipide A sont obtenues. Il est particulièrement intéressant de remarquer que des lipides A dérivés de lipopolysaccharides eux-même inactifs deviennent hautement actifs après solubilisation par l'albumine du sérum de boeuf.

Rôle des anticorps naturels (Publication No.III)

Le sérum de cobaye utilisé pour ces études comme source de complément renferme un grand nombre de spécificités anti-lipopolysaccharidiques différents. Ces anticorps furent mis en évidence dans le test d'hémolyse passive. On utilise des globules rouges de mouton sensibilisés par la préparation après hydrolyse alcaline de chaque lipopolysaccharide testé. 52 des 54 lipopolysaccharides testés donnèrent des titres d'hémolyse allant de 1:8 à 1:128.

Le mécanisme de l'activité anti-complémentaire des lipopolysaccharides n'a pas pu être expliqué jusqu'ici. Si on ne tient pas compte des autres mécanismes participant à l'interaction lipopolysaccharide-complément, ces résultats montrent toujours la présence d'un mécanisme spécifique utilisant les anticorps naturels comme médiateurs. Etant donné que la quantité seule de ces anticorps est suffisante pour être responsable des activités biologiques variées résultant de cette interaction, il faut être prudent quant à la signification biologique des autres mécanismes non immunologiques qui y participent.

Endotoxicité du lipide A (Publications No. V,VI)

Le lipide A est la partie de la molécule lipopolysaccharidique responsable de l'endotoxicité. Le lipide A solubilisé par l'albumine du sérum de boeuf est capable de reproduire un grand nombre des activités de l'endotoxine. Celles-ci comportent entre autres l'effet létal chez des souris normales, surrénalectomisées ou sensibilisées par le BCG, la pyrogénicité chez les lapins, la résistance non-spécifique à l'infection. Dans les tests ci-dessus l'activité des complexes lipide A/BSA était comparable à celle de l'endotoxine intacte.

D'autres activités biologiques des complexes lipide A/BSA furent testées dans de nombreux laboratoires. Le lipide A utilisé dans les tests ci-dessus était totalement dépourvu de polysaccharide. On peut en conclure que le lipide A est le principe actif du lipopolysaccharide. La partie polysaccha-

ridique sert d'agent solubilisant au lipide A normalement insoluble. Sa présence n'est pas essentielle à l'expression de l'activité biologique. Elle peut être remplacée par d'autres agents solubilisant comme l'albumine du sérum de boeuf citée plus haut.

Immunogénicité du lipide A (Publications No. VII, VIII)

Quand les animaux sont immunisés avec des bactéries entières, les anticorps sont formés avant tout contre les chaînes polysaccharidiques bien exposées. A cause de sa position plus profonde, le lipide A n'est pas un bon immunogène.

On a pu rendre le lipide A immunogène, en l'exposant à la surface de la cellule bactérienne. Ceci est réalisé grâce à l'hydrolyse acide de la bactérie qui détache le polysaccharide suivie d'une fixation supplémentaire de lipide A libre à la surface de la cellule bactérienne. Ces cellules enrichies en lipide A, se montrèrent de bons immunogènes chez les lapins et les chèvres avec production de sérum anti-lipide A de titres élevés.

Les anticorps dirigés contre le lipide A sont très spécifiques pour ce dernier et appartiennent à la fois aux classes IgM et IgG. Ils réagissent avec la partie lipide A d'un grand nombre des lipopolysaccharides sérologiquement différents et opsonisent les bactéries E.coli 0111 pour la phagocytose par les cellules péritonéales chez la souris.

Les anticorps anti-lipide A apparaissent naturellement dans le sérum de nombreux animaux et chez la plupart des humains. Leur signification biologique n'est pas connue jusque ici. Leur rôle pourrait être protecteur, nuisible ou les deux.

INTRODUCTION

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Lipopolysaccharides are constituents of the outer cell-wall of gram-negative bacteria (1). They are toxic substances being responsible for many of the biological activities associated with gram-negative infections (2,3,4,5). During the life and growth of bacteria, these substances are generally kept on the cell and are not released in the environment. The release of lipopolysaccharides from bacteria is usually associated with the death and lysis of the cell. So in contrast to the exotoxins which are released from living cells these substances were named endotoxins. At the same time lipopolysaccharides represent the somatic antigens of gram-negative bacteria. Immunization of animals or humans with live or killed bacteria leads to the production of antibodies which are directed against structures in the lipopolysaccharide molecule.

General structure of bacterial lipopolysaccharides

Lipopolysaccharides are made up of two parts of contrasting chemical and physical properties. A hydrophilic sugar part, the polysaccharide and a hydrophobic lipid part, the lipid A (see Fig. 1). In the polysaccharide part two distinct regions can be recognized, the basal core polysaccharide and the O-specific polysaccharide.



Fig. 1: The three regions in the lipopolysaccharide molecule (LÜDERITZ 1970) (6).

O-Polysaccharide

The O-polysaccharide represents the variable part of the lipopolysaccharide molecule. It is made up of repeating oligosaccharide

units which vary in number but usually about 8-12. Its sugar composition and thereby serological specificity is different among the various species. This part of the molecule is the carrier of the O-antigenic specificity of the lipopolysaccharide and it is by the serological reactivity of this region that lipopolysaccharides were classified into serotypes (7, 8).

Basal core polysaccharide (9, 10)

This region is identical in all the species within a genus. In the case of *Salmonella* it is made up of a decasaccharide in which 5 different sugars may be recognized. These are GlcNAc, Glc, Gal, Hep and KDO, the last one being present in the form of a branched trisaccharide (see Fig. 2). There are also other constituents present in the core PS in small quantities. Thus one of the heptoses carries a P-PEN and a PEN is present in one of the 3 KDO residues. The core polysaccharide forms the bridge between the O-polysaccharide and lipid A.

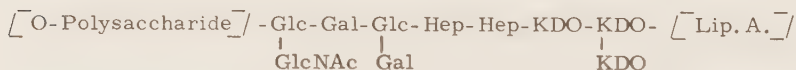


Fig. 2: Structure of the core polysaccharide
(LÜDERITZ 1970) (6).

Lipid A

This region is a common constituent of the lipopolysaccharides of all gram-negative bacteria. Its structure is very similar if not identical among *Enterobacteriaceae* (11, 12). It is made up of a glucosaminyl- β -1, 6 glucosamine disaccharide which is often referred to as the back-bone of lipid A (13). The amino groups of this disaccharide are substituted by β -hydroxymyristic acid while the hydroxyl groups are esterified with equimolar amounts of palmitic, lauric and 3-myristoxymyristic

acid. Very recent results obtained by LEHMAN et al. provide evidence that such diglucosamine units are polymerized via pyrophosphate bridges at positions 1 and 4. The formula in Fig. 3 shows the structure of lipid A according to our present knowledge.

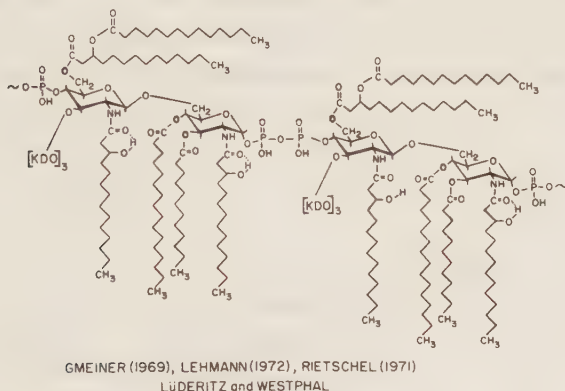


Fig. 3: Structure of lipid A

The rough(R) mutants of gram-negative bacteria

These are derived from the wild(S) forms as a result of one or more genetic defects. They synthesize lipopolysaccharides in which the O-specific chains are absent and the core polysaccharide is present in a more or less incomplete form according to the point of defect (14).

The classification of these mutants into the various R-classes is based on the chemical composition of the core polysaccharide and is dependent upon the number of core specific sugars still present in the molecule (15).

There are six classes of R-mutants designated: Ra, Rb, Rc, Rd₁, Rd₂ and Re. Mutants belonging to the class Ra synthesize a complete core polysaccharide. Those belonging to class Rb synthesize an incomplete core from which terminal N-acetylgluco-

samine is absent. Further defects in the synthesis of galactose, glucose and heptose result in the classes Rc, Rd, and Re, respectively. The last class accommodates the most incomplete mutants. Their lipopolysaccharides contain besides lipid A, only three KDO residues as the last remnants of the core polysaccharide. Mutants defective also in the synthesis of KDO are as yet unknown. Fig. 4 shows in a semi-schematic representation the structure of the lipopolysaccharide molecule in its complete form and that of the various defective lipopolysaccharides of the R mutants.

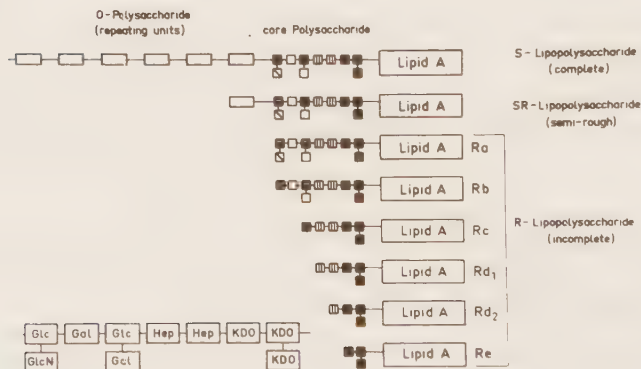


Fig. 4: Schematic representation of the structure of S- and R-form lipopolysaccharides.

Isolation of lipopolysaccharides

There exist several methods for the isolation of lipopolysaccharides from gram-negative bacteria (16). Of these, two are mentioned because of their relevance to the work presented here. The first is the

trichloroacetic acid procedure (17) and the second the phenol-water method (18). By the former method the lipopolysaccharide is obtained in complex form with protein the amount of which varies from 10% to 20%. The phenol-water method yields lipopolysaccharides in fairly pure form and has a wide range of applicability. The extracts obtained by this method are usually free of nucleic acid and the amount of protein present is fairly low usually 1% to 3%. All methods described so far were originally intended for the isolation of smooth-form lipopolysaccharides. Their application for the extraction of R-form lipopolysaccharides is often unsatisfactory and in many cases low or no yields of lipopolysaccharides may be expected.

RESULTS

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At about the time when this work commenced, the first rough mutant strains of gram-negative bacteria were isolated. At that time the principal interest in our laboratory was the chemical structure and serological reactivity of the lipopolysaccharides of these deficient mutants. My own task was to identify the serological determinants in the lipopolysaccharides of various R mutants by studying the inhibitory activity of their oligosaccharides obtained by partial acid hydrolysis of the lipopolysaccharide. These studies proved difficult because the yield of oligosaccharides obtained was so low (0,3%) that it was practically consumed by analytical studies. The poor yields of oligosaccharides has always been a major problem when dealing with partial acid degradation of heteropolymers containing linkages of various degree of lability. A considerable amount of work was thus devoted to the study of hydrolysis conditions under which improved oligosaccharide yields could be obtained. These studies led to the development of the hydrolysis method described below.

A new method of hydrolysis of lipopolysaccharides (Publication No. I)

The apparatus used is shown in the diagram of Fig. 5. It consists of a heating chamber, a cooling chamber and a dialysis chamber. The set up is a closed circuit and the flow in this circuit is effected by a peristaltic pump. The lipopolysaccharide in acid solution first enters the hydrolysis chamber where it is heated for a short time. It then enters the cooling chamber where hydrolysis stops. It is subsequently passed through a dialysis chamber where small dialysable molecules are removed (19) and adsorbed onto a cooled charcoal column thus protected from further degradation.

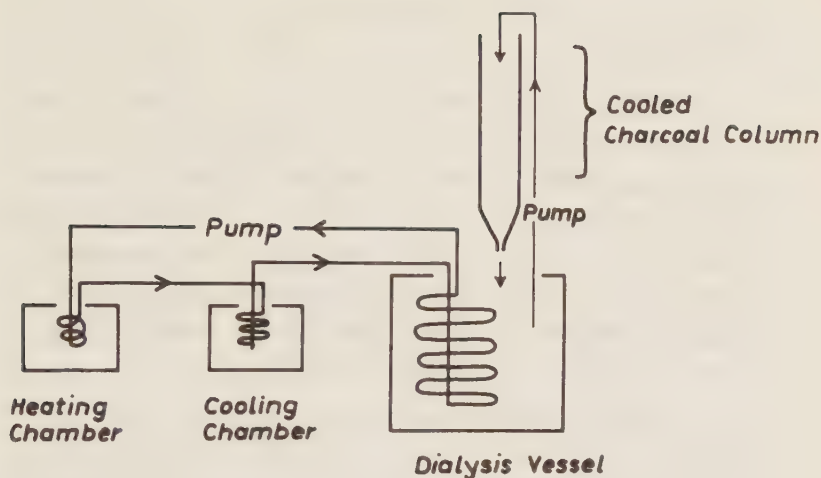


Fig. 5: Continuous Hydrolysis Apparatus

The hydrolysate solution then returns to the heating chamber for further hydrolysis. Under these conditions small oligosaccharide molecules formed are immediately cooled, removed from the hydrolysate and adsorbed onto a cooled charcoal column thus protected from further degradation. The method is very flexible because the conditions (length of heating and dialysis period, strength of acid and temperature) can all be independently varied to suit the hydrolytic properties of the polymer in question. The method proved successful and has since been in routine use in our laboratory and elsewhere.

A new method, specific for the extraction of R-form lipopoly-saccharides (Publication No. II)

The lipopolysaccharides of R-mutants presented a new class with characteristic physical properties. It was felt that a new method

was needed for isolating these substances in optimum yields and in a pure form.

The loss of the O-specific polysaccharide in the R-mutants means a large reduction in the number of polar groups in the molecule and because the lipid moiety remains unaltered the molecule thus acquires a strongly lipophilic character. It seemed reasonable to assume that solubility in water decreases and that in organic solvents increases. With these considerations in mind, I was prompted to look for an organic solvent system suitable for the extraction of R-form lipopolysaccharides.

In the course of my work on R-form lipopolysaccharides it was observed that these were fairly soluble in mixtures of aqueous phenol and chloroform. These mixtures, however, could also dissolve proteins but to a lesser extent. A third solvent was then searched for, which when added to the mixture of phenol and chloroform could still extract R-form lipopolysaccharide but not the other contaminants. This was achieved with petroleum ether (bp. 40-60°). By adjusting the ratio of phenol:chloroform:petroleum ether to 2:5:8, a monophasic mixture was obtained which proved suitable for the isolation of R-form lipopolysaccharides from bacteria. The yields obtained by this procedure are generally high and the extracts are free of nucleic acid and proteins. The method proved very successful and to-day it is in routine use in our laboratory and elsewhere.

Further purification of lipopolysaccharides (Publication No. III)

Apart from proteins and other macromolecules, lipopolysaccharides are invariably contaminated with divalent ions, like Mg^{++} and Ca^{++} and basic amines such as ethanolamine, spermine, spermidine and putrescin (20). Whatever the extraction procedure employed these

substances are always present in the lipopolysaccharide and their removal has been difficult. Recently, a procedure for their removal has been developed which is based on the principle of electrodialysis. The apparatus employed is made up of three compartments separated from one another by two dialysis membranes. The two outer chambers are equipped with electrodes and are filled with distilled water. The middle compartment contains the lipopolysaccharide dissolved in distilled water. A potential difference is applied to the system (50-200 v), and after a few min. the pH of the contents of the cathode chamber begins to rise, indicating that basic material has diffused in from the middle chamber. The rise in the pH of the cathode chamber is paralleled by a drop in the pH of the middle chamber containing the lipopolysaccharide. At the same time it can be seen that the lipopolysaccharide which was originally present in a soluble form separates from the water in very fine gel particles, migrating slowly towards the anode membrane where it deposits. When the pH of the cathode chamber rises above 11 its contents are replaced by fresh distilled water. This is repeated several times until no further rise in pH takes place. Throughout the experiment the pH in the anode chamber remains unchanged, showing that no acidic material of low molecular weight has separated from the lipopolysaccharide. At the end of the experiment the chambers are emptied and the lipopolysaccharide is collected by scraping it off from the anode membrane. Electrodialysed lipopolysaccharide is in the free acid form and this results in decreased solubility. It can be made again completely soluble by converting into the Na^+ or K^+ form with the respective alkali or by neutralization with any of the basic amines mentioned above.

A large number of different S- and R-form lipopolysaccharides have been subjected to electrodialysis and in all cases similar results were obtained. After treatment, all preparations became acidic and

migrated to the anode. In all cases only basic material separated from lipopolysaccharides.

Effect of electro dialysis on the physical properties of lipopolysaccharides

Removal of Mg^{++} and Ca^{++} and of basic amines from the lipopolysaccharide and conversion to the triethylamine salt-form, results in the extensive disaggregation of the lipopolysaccharide. Fig. 6 shows the sedimentation pattern of several S- and R-form lipopolysaccharides before and after electro dialysis. In all cases the sedimentation velocity of the various preparations is much lower than that of the corresponding preparation before electro dialysis.

After electro dialysis lipopolysaccharides become acidic and undergo autolysis when heated in distilled water .

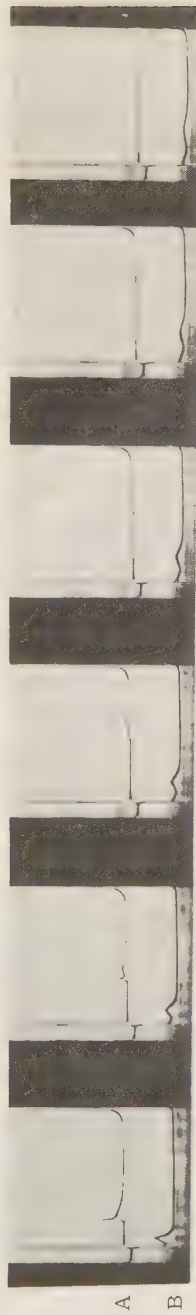
The lipid A which has so far been known as a completely water insoluble material, shows increased solubility when prepared from electro dialysed lipopolysaccharides.

Sedimentation Properties of Lipopolysaccharides after Electrodialysis

S. minn. R5



S. typhim.
TV 161



S. ab. equi.

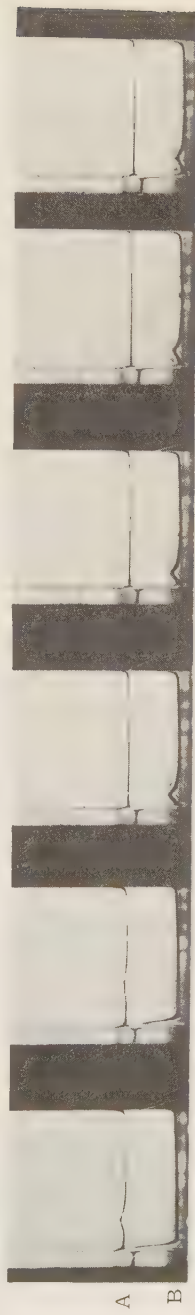


Fig. 6 : A = before electrodialysis, B = after electrodialysis.

BIOLOGICAL ACTIVITIES OF LIPOPOLYSACCHARIDES

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Injection of purified endotoxic lipopolysaccharides (ET) in experimental animals sparks off a number of biological activities which resemble those produced during an infection with gram-negative micro-organisms (5). The mechanisms underlying these activities are as yet unknown. However, they may be placed in two categories. The specific and the non-specific ones. The non-specific action of ET represents primary activity. In this case ET acts directly on cellular and humoral mechanisms and these actions are not dependent on the host's immunological predisposition to endotoxin. The specific action of ET represents secondary activity and is of an immunological nature. It proceeds only in animals which have been primed to ET by previous encounter of the antigen. Since the animal or human organism is confronted with gram-negative bacteria continuously from a very early stage in life, it becomes very difficult in many cases to decide whether some of the biological activities of ET's represent primary or secondary responses.

One such case in which it has been very difficult to decide between a primary or secondary activity is the in vitro action of endotoxin on complement. Complement is a serum constituent, made up of 9 protein components. Complement has the property of interacting with antigen/antibody complexes whereby the 9 components are sequentially activated, leading to a number of biological activities, the classical among these being lysis of cells. For a long time it has been known that lipopolysaccharides can also interact with complement; in this case, however, also in the absence of added antibody. PILLEMER was the first to show that when endotoxin is added to fresh guinea pig serum in vitro there follows a marked loss of hemolytic complement activity (21). More recent

studies by MERGENHAGEN, GEWURZ and others (22), showed that during the interaction of lipopolysaccharides with complement, terminal complement activity mainly C_3 is consumed with little or no consumption of the early acting components $C_{1,4,2}$, which always proceeds during the classical activation of complement by Ag/Ab complexes. This mechanism which excludes the participation of the early acting components is known as the "alternate pathway" of complement activation. The possible biological significance of the in vitro complement/lipopolysaccharide interaction lies in the fact that a number of activities are generated which are known to follow in animals which were administered ET. (22) These include platelet aggregation, generation of anaphylatoxin and of chemotactic factor, depression of blood pressure, and reports appear constantly describing new complement dependent endotoxic activities. The present study will present results which show that anti-complementary activity is not a property expressed by all lipopolysaccharides. Further it will be shown that lipid A is the part of the lipopolysaccharide molecule responsible for anti-complementary activity. Finally the role of natural antibodies in the lipopolysaccharide/complement interaction is investigated.

Anti-complementary activity of lipopolysaccharides (Publication No. IV)

Anti-complementary activity is a property expressed only by soluble lipopolysaccharides. Insoluble preparations exhibit only negligible activity which however becomes very high if specific antibody is added. The effect of the divalent cations Mg^{++} and Ca^{++} on the anti-complementary activity of lipopolysaccharides has been studied. It was found that these ions which are known to precipitate R-form lipopolysaccharides from their solutions also abolish their anti-complementary activity. S-form lipopolysaccharides on the other

hand are less susceptible to the precipitating effect of these ions and their activity remains practically unaffected by divalent ion concentrations which usually render R-form lipopolysaccharides completely inactive.

Although solubility of a lipopolysaccharide is necessary for the expression of anti-complementary activity, not all soluble lipopolysaccharides are anti-complementary. Screening of a large number of soluble smooth and rough form lipopolysaccharides obtained from Salmonella, Arizona and E.coli (Fig. 7) showed that only about one third of the preparations are highly anti-complementary. The activity of the others varied from medium to practically no activity at all. The pattern of complement inactivation of the lipopolysaccharides tested, falls into one of three types represented by the curves A, B and C of miniature Fig. 7 a. With lipopolysaccharides showing curve A, 100% complement inactivation can be obtained, which is not lowered by excessive amounts of lipopolysaccharide. With lipopolysaccharides showing curve B, a maximum loss of complement activity is obtained, (which is less than 100%) by a given amount of lipopolysaccharide and addition of more results in less complement being fixed so that 100% inactivation is not obtainable. Preparations with curve C inactivate complement only to an insignificant extent, no matter how much lipopolysaccharide is added. Therefore, the difference between active and less active lipopolysaccharides is a qualitative and not a quantitative one.

The reason why some lipopolysaccharides are anti-complementary and others are not, is not yet known. On the other hand it seems that the anti-complementary activity of all lipopolysaccharides of Enterobacteriaceae falls into one of the above groups A, B, C. They are related neither to the genus of bacteria from which the lipopolysaccharides derive nor to the class (S or R) to which they belong; i. e.

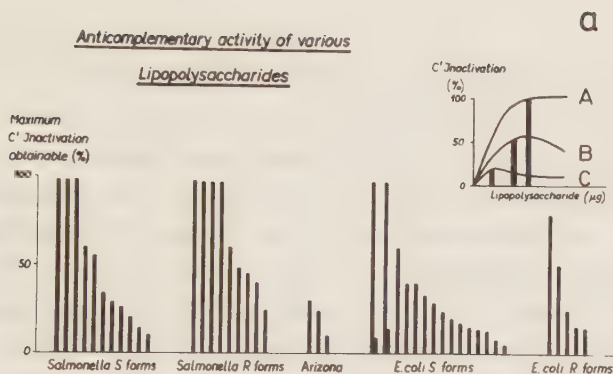


Fig. 7

whether a lipopolysaccharide can inactivate complement or not is independent of the quantity or quality of sugars that are present in the molecule. Thus the high anti-complementary activity of some R-form lipopolysaccharides provides conclusive evidence that the O-specific polysaccharide region is not necessary for anti-complementary activity. Further, since the most defective lipopolysaccharide, mR595 which contains only lipid A and KDO as the only sugar is highly anti-complementary, it also becomes evident that the largest part of the core polysaccharide plays no role in the anti-complementary activity.

One more important information results from this survey: All the lipopolysaccharides tested here are highly active as endo-

toxins, therefore, the toxicity of a lipopolysaccharide and its *in vitro* anti-complementary activity do not always run parallel.

The role of KDO in anti-complementary activity of lipopolysaccharide (Publication No. V.)

The requirement for KDO in anti-complementary activity and generally biological activity of a lipopolysaccharide has been the subject of considerable speculation the last 5 years. This speculation was not completely unjustified: This unusual 8 carbon sugar is a common constituent of lipopolysaccharides of all enterobacteriaceae. Also its cleavage from the lipid A by acid hydrolysis was always paralleled by loss of biological activity. (23)

In the mR595 lipopolysaccharide (class Re), KDO acts as immunodominant. To study the role of this sugar in anti-complementary activity, a series of chemical treatments was carried out which completely abolished the original serological specificity of the lipopolysaccharide. These included substitution of the hydroxyl groups with succinyl residues by treating with succinic anhydride. (24) A second treatment consisted of converting the carboxyl group of KDO to the methyl ester forms by treating with diazomethane (24). (For details see RIETSCHER et al. in this thesis). Both treatments resulted in the complete loss of the original serological specificity of the lipopolysaccharide, as tested by the passive hemagglutination test. In the complement fixation test, however, the anti-complementary activity of the two derivatives was found unaltered. From these results RIETSCHER et al. concluded that the presence of KDO in the lipopolysaccharide molecule is not necessary for anti-complementary activity.

Lipid A as the anti-complementary principle of lipopolysaccharides (Publication No.IV, V)

Soluble lipid A/BSA complexes

So far, by employing defective lipopolysaccharides, it could be shown that the O-specific polysaccharide chains as well as part of the core polysaccharide are not necessary for anti-complementary activity. Using chemically modified R595 lipopolysaccharide the possible role of KDO in anti-complementary activity could also be eliminated. These results pointed very strongly to lipid A as being responsible for anti-complementary activity. Direct evidence, however, could not be obtained because free lipid A prepared from lipopolysaccharide by acid hydrolysis is completely insoluble in water and is thus inactive. Solubilization of lipid A was achieved by complexation with soluble carriers.

Lipopolysaccharides have a high affinity for proteins, lipids and other lipopolysaccharides. This affinity is due to the presence of lipid A in the molecule. We made use of this property to complex lipid A with solubilizing carriers which themselves exhibit no anti-complementary activity. Three different carriers were employed, one of which is bovine serum albumin (BSA). Complexation of lipid A with BSA was carried out according to the scheme of Fig. 8. Lipid A is dissolved in water by addition of triethylamine and mixed with a solution of the carrier. Complexation is allowed to take place by expelling the triethylamine under vacuum, whereby a water-soluble complex is obtained. Several lipid A preparations were complexed with BSA and subjected to anti-complementary activity tests. Although the various preparations were derived from lipopolysacchyrdes of varying anti-complementary activity (group A,B,C above), the activity of the complexes was found to

Preparation of Watersoluble
Lipid A • BSA Complexes

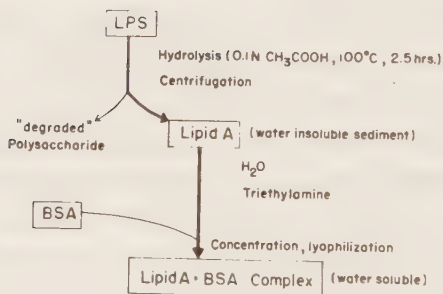


Fig. 8:

be in all cases very much the same. This shows that the activity of isolated lipid A/carrier complexes is independent of the activity of the parent lipopolysaccharide from which the various lipid A preparations were obtained. Of particular interest is the fact that highly anti-complementary complexes were also obtained with lipid A derived from lipopolysaccharides which themselves exhibit no anti-complementary activity. From these results it is concluded that the ability of a lipopolysaccharide to inactivate complement is embedded in the lipid A part of the molecule but for some unknown reason in some lipopolysaccharides this is not expressed. The polysaccharide part as such plays no role in anti-complementary activity. It only functions as a solubilizing carrier for the insoluble lipid A and may be replaced by other carriers.

The Role of Natural Antibodies in the Lipopolysaccharide/
Complement Interaction (Publication No. III)

When discussing the interaction of lipopolysaccharide with complement (in the absence of added antibody) one is always confronted with the inevitable question concerning natural antibodies which may be present in the serum used as source of complement. The shape of curves B and C of Fig. 7a are very reminiscent of the pattern of complement fixation by antigen/antibody complexes. This prompted us to investigate the level of natural anti-lipopolysaccharide activity present in the guinea pig serum that we used as complement source.

To determine natural antibodies the passive hemolysis test was used with sheep red cells (SRC) passively sensitized with the respective lipopolysaccharides. All lipopolysaccharides used were partly deesterified by heating in 0.25 N NaOH at 56°/1 hr. This treatment inactivates lipopolysaccharides and leads to loss of anti-complementary activity (22). In this way, direct interaction of lipopolysaccharide with complement could be excluded. The guinea pig serum was absorbed in the cold with SRC to remove any anti-SRC activity present. The tests were carried out in large hemagglutination plates. Serial dilutions of the guinea pig serum were made, sensitized SRC were added and the plates incubated at 37°/1 hr. Lysis of the lipopolysaccharide-coated cells is interpreted as antibody activity present in the serum. The highest dilution of guinea pig serum at which lysis of the SRC still occurs, represents the hemolytic end titre of the serum towards the lipopolysaccharide in question. The results are shown in Fig. 9. Hemolytic titres ranging from 1/8 to 1/128 could be demonstrated with 52 out of the 54 lipopolysaccharides tested. These lipopolysaccharides were randomly chosen and included both S- and R-form preparations obtained from a variety of Salmonella, Arizona and E. coli strains.

Anti-lipopolysaccharide activity present in pooled normal guinea pig serum

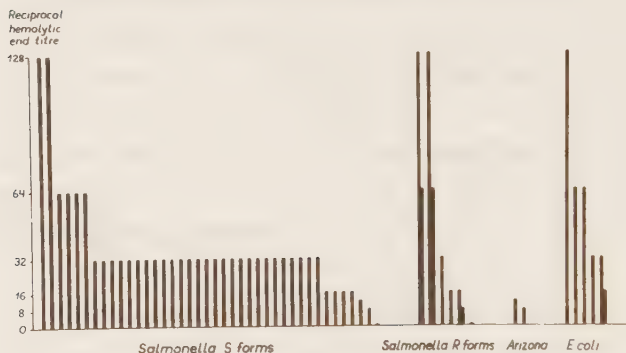


Fig. 9:

To show that the activities obtained were due to specific anti-lipopolysaccharide antibodies, absorption tests were carried out as follows. Six lipopolysaccharides were chosen randomly and their respective antibody titre in the guinea pig serum was determined. Serum samples were then adsorbed with each of the six lipopolysaccharides fixed to SRC and each absorbed serum was tested once more with all six lipopolysaccharides. In each case only the activity to the lipopolysaccharide with which the serum was absorbed disappeared while the activity against the other lipopolysaccharides remained either unaltered or only slightly diminished.

The results of the above experiments show that the hemolytic titres obtained do not represent activation of the complement system by lipopolysaccharide/complement interaction. This can be ruled out because the lipopolysaccharide preparations employed were alkali-treated which are known to be inactive with respect to complement fixation. This is supported further by the results of the ab-

sorption experiments where absorption of the serum with one lipopolysaccharide leads to loss of hemolytic activity against this lipopolysaccharide only.

The conclusions I would like to draw from these results are the following: Pooled guinea pig serum which is used universally as a source of complement contains a large number of anti-lipopolysaccharide specificities. Thus on addition of lipopolysaccharide to complement part of the lost hemolytic activity measured is almost always due to specific natural antibody/lipopolysaccharide interaction.

Also in the case of the lipid A/complement interaction antibodies may be playing a role as these have been found to exist naturally in the serum of animals and humans. (Publication No. III)

At this point I would like to postulate a mechanism which has been virtually overlooked and which must be certainly playing at least a part role in the anti-complementary activity of lipopolysaccharides. As mentioned earlier lipopolysaccharides have a high affinity for proteins with which they form complexes, and there is no reason why all 9 proteins of the complement system should form an exception. Thus on addition of lipopolysaccharide to complement, absorption of one or more complement components by the lipopolysaccharide may take place, leading to partial loss of hemolytic activity.

More than one mechanisms may thus be involved in the lipopolysaccharide/complement interaction, and at the moment the various activities that result from this interaction may only be viewed upon as the collective result of different mechanisms.

ENDOTOXICITY

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(Part of the work presented hereafter was carried out in the laboratories of Dr. Chedid, the Institute Pasteur, Paris)

Lipid A, the biologically active region of lipopolysaccharides

In the previous chapter it was shown that free lipid A isolated by acid hydrolysis of the lipopolysaccharide is highly anti-complementary when made soluble by complexation with BSA. The present chapter will deal with the endotoxic and generally biological properties of lipid A/BSA complexes. It will be shown that many biological activities which are manifested by endotoxin can be reproduced with free lipid A complexed with BSA.

Lethal toxicity in normal mice

A number of lipid A preparations obtained from different lipopolysaccharides by acetic acid hydrolysis were complexed with BSA in the ratio of 1:1 (wt/wt) according to the procedure described earlier. They were injected intraperitoneally into NMRI white mice, weighing approximately 20 gm and deaths were recorded up to 48 hrs. later. The results are shown in Tab. 1 (first column). The toxicity of these complexes is comparable to that of the mR595 Re glycolipid, and the LD50 lies between 1500-4000 μ g.

Biological properties of Lipid A/BSA complexes as compared with the parent lipopolysaccharides (glycolipids)

Preparation	Mouse lethality LD ₅₀ (μ g)	Pyrogenicity MPO-3 (μ g/kg)	Bone marrow reaction ED ₅₀ (μ g)	Complement inactivation % inhibition by 10 μ g LPS or LipA/BSA	Limulus lysate gelation (μ g/ml)
<i>S. minnesota</i> R60 LPS	300			30	
LipA/BSA	1700	0,003	18,8	86	
<i>S. minnesota</i> R345 LPS	300		6,2	5	
LipA/BSA	1700	0,013	7,2	85	10 ⁻⁸
<i>S. minnesota</i> R5 LPS	650			70	
LipA/BSA	4000	0,007	10,9	85	10 ⁻¹²
<i>S. minnesota</i> R595 LPS	1750	0,004	3,9	80	
LipA/BSA	4000	0,007	11,0	86	10 ⁻¹²
<i>E. coli</i> EH700 LPS				15	
LipA/BSA	1700			80	10 ⁻⁶
BSA control	>10 000	>100	>100	>100	

Tab. 1

Lethal Toxicity in adrenalectomized mice

The corticosteroids produced in the adrenal glands antagonise the action of endotoxin. Therefore, when mice are subjected to adrenalectomy they are rendered highly sensitive to the lethal effects of endotoxin. The amount of endotoxin necessary to kill an adrenalectomized mouse is about ten thousand-fold lower than that necessary to kill a normal animal (25-28). Adrenalectomy thus represents an extremely sensitive system for testing the lethal effects of endotoxin in mice, and it was adopted here for comparing the toxicity of lipid A with a number of lipopolysaccharides and boivin extracted preparations. Swiss albino mice were used 48 hrs. after adrenalectomy (28). The various endotoxin preparations were administered intravenously and deaths were recorded up to 48 hrs. thereafter. The following preparations were tested: Boivin-extracted endotoxin from *S. enteritidis* a phenol/water preparation of *S. minnesota* S-form and the glycolipids of *S. minnesota* R345 and R595 obtained by the PCP extraction method. The lipid A was used in complex form with BSA and derived from the lipopolysaccharide of the *Salmonella* mutant R345. In order to increase the solubility and thereby obtain a higher degree of molecular dispersion, the lipid A/BSA complex was treated with Sodium deoxycholate (NaD) and then dialyzed against distilled water. The toxicity of both the deoxycholate and of the non-treated complex were tested. The results are shown in Tab. 2. The LD_{50} obtained by lipid A/BSA was 0.2 μg . After sodium deoxycholate treatment, however, a LD_{50} of 0.02 μg was obtained which is higher than that of the lipopolysaccharides tested and equal to that of the Boivin extracted preparation.

Toxicity of Lipid A/BSA Complex and Lipopolysaccharides in

Adrenalectomized Mice

	<u>LD₅₀ (μg)</u>
<i>S. enteriditis</i> (boivin extract)	0,02
<i>S. minnesota</i> S form LPS (phenol/water)	0,06
<i>S. minnesota</i> R345 (Rb)	0,075
<i>S. minnesota</i> R595 (Re)	0,14
Lipid A/BSA	0,3
Lipid A/BSA (further dispersed by NaD)	0,02

(M. PARANT and C. GALANOS)

Tab. 2

Non-specific resistance to infection

Administration of microgram amounts of endotoxin renders mice resistant to lethal doses of infective micro-organisms (29). The ability of free lipid A to induce non-specific resistance to infection in mice was studied and compared with a Boivin, and 2 phenol/water endotoxin preparations.

Mice were injected intravenously with various amounts of the above preparations, and 24 hours later they were challenged with a lethal dose (10^5 cells) of *Klebsiella pneumoniae*. Table 3 shows the results obtained. All control animals died within the first 24 hours. 100% protection was obtained by all preparations including the lipid BSA complex and the soluble lipid A preparation. From these results it is concluded that free lipid A, like whole endotoxin is capable of inducing non-specific resistance to infection.

EFFECT OF LIPID A ON THE NON-SPECIFIC RESISTANCE
TO INFECTION BY KLEPSIELLA PNEUMONIA.

	MORTALITY			
	24HR.	48HR.	5DAYS	6DAYS
CONTROLS	7/8	7/8	8/8	8/8
MR595 LPS 1 μ G	0/8	0/8	0/8	0/8
LIP A/BSA 1 μ G	0/8	0/8	0/8	0/8
SOLUBLE LIPID A 1 μ G	0/8	0/8	0/8	0/8
LIP A OH 1 μ G	0/8	0/8	1/8	2/8

THE VARIOUS PREPARATIONS WERE INJECTED INTRAVENOUSLY IN MICE,
24 HRS PRIOR TO INFECTION.

Tab. 3

Sensitization of mice to lipid A by BCG

Another characteristic activity of lipopolysaccharide is that their lethal toxicity is considerably increased in animals that had been treated with BCG. (30-32) This property of increased activity could also be demonstrated with lipid A complexed to BSA (Table 4).

In normal mice, lipid A/BSA complex is usually lethal in concentrations of 1 to 1.5 mg. In BCG-treated mice, however, its toxicity is enhanced and the animals already die with concentrations of 5 μ g.

SENSITIZATION OF MICE TO LIPID A BY BCG.

300 μ g* BCG WAS ADMINISTERED INTRAVENOUSLY IN AKXDBA/T6 MICE AND 14 DAYS LATER THE ANIMALS WERE CHALLENGED WITH LIPID A, MR595 LPS, AND S. ENTERIDITIS BOIVIN EXTRACTED ENDOTOXIN.

	DOSE (μ g)	MORTALITY AFTER 48 HR.
S. ENTERIDITIS ENDOTOXIN	1.5	2/5
	5	4/5
	15	5/5
	50	4/5
MR595 LPS	0.5	1/8
	1.5	4/8
	5	3/8
	15	6/8
	50	6/8
LIP A/BSA (NAD)	1.5	1/8
	5	3/8
	15	6/8
	50	6/8

Tab. 4

Pyrogenicity (Publication No. VI)

Of the many biological activities of endotoxins, pyrogenicity is perhaps their most characteristic property. Injection of sub-microgram amounts of lipopolysaccharide in rabbits leads to the development of fever with a biphasic pattern, with maximum temperatures at 1 and 3 hrs. after injection. (33)

The pyrogenicity of various lipid A/BSA complexes have been tested in comparison with the respective lipopolysaccharides from which the lipid A was obtained. Tab. 5 shows the results obtained. All lipid A/BSA preparations proved highly pyrogenic with an activity comparable to that of the respective parent lipopolysaccharide.

Lipopolysaccharide or Lipid A · Albumin Complex			Pyrogenicity MPD-3
			(µg/kg)
S. minnesota	R595	LPS	0.005
		Lipid A · BSA	0.005, 0.007
S. minnesota	R5	LPS	n. d.
		Lipid A · BSA	0.007
S. minnesota	R345	LPS	0.0007
		Lipid A · BSA	0.001
S. minnesota	R60	LPS	n. d.
		Lipid A · BSA	0.003
Albumin Controls		BSA	100

Table 5: Pyrogenicity of various lipid A · Albumin complexes as compared with the parent lipopolysaccharide

The present results show that many biological activities expressed by whole endotoxin can be reproduced with free lipid A in complex form with BSA. Further biological activities of lipid A/BSA complexes have also been demonstrated in/or in collaboration with other laboratories. These include, interferon production (34), tumour necrosis (35), bone marrow necrosis (36) and mitogenic activity (37, 38, 39). The lipid A employed in all the above studies was completely free of polysaccharide as judged by the absence of KDO. It is therefore concluded that lipid A is the biologically active principle of lipopolysaccharides.

The polysaccharide acts as a solubilizing carrier for the insoluble lipid A. Its presence, however, is not essential for the expression of toxicity and it may be replaced by other solubilizing carriers, as in the above cases by BSA.

IMMUNOLOGICAL PROPERTIES OF LIPID A

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Immunization of animals with whole bacteria or isolated lipopolysaccharide leads to the production of antibodies which in the past were always found to be directed against the polysaccharide part of the lipopolysaccharide molecule. Although the presence of anti-lipid A antibodies was often strongly suspected, the role of lipid A as an antigen remained virtually unexplored. There are two main reasons for this. The first was the prejudicial assumption that lipid A was bound to be non-immunogenic because lipids generally are. A more serious reason which discouraged many workers from embarking on immunological studies on lipid A was that until about 5 years ago there was virtually very little known about the chemical structure and composition of this material. Thus for a long time it represented a dark mysterious region in the lipopolysaccharide, and its description as "Black Box" would have been quite appropriate had it been applied a few years earlier. The last 5 years, however, thanks to the work of GMEINER et al. (40), RIETSCHEL et al. (41), and LEHMAN et al. (42) a large amount of information has been obtained concerning its structure and composition and to-day we have a fairly good idea of what lipid A looks like.

There are also other reasons which kept immunogenic studies on lipid A in the background. Its total lack of solubility and its non-specific interaction with proteins makes studies on lipid A as an antigen very tedious. Thus we know to-day, that the usual serological tests such as precipitation, hemagglutination or gel precipitation which are the commonest tools for preliminary evaluation of a specific antibody response to an antigen are unsuitable methods for identifying anti-lipid A antibodies, especially when the latter are present in small concentrations. However, after having demonstrated

that lipid A is the toxic principle of lipopolysaccharides, we felt that the time was ripe to take a look at the question concerning its immunogenicity.

Preparation of immunogenic Lipid A (Publication No. VII, VIII)

If we could view at the lipopolysaccharide in its native form as it appears on the bacterial cell, we would probably recognize that the O-specific chains and the core polysaccharide protrude outwards into the surrounding medium while the lipid is embedded in the cell-wall and forms part of its rigid structure.

When animals are immunized with whole bacteria, antibodies are readily produced to the well exposed polysaccharide chains. Lipid A, on the other hand because of its cryptic position, is very poorly exposed to act as a potent immunogen.

The first step in obtaining lipid A in an immunogenic form was to expose it on the surface of the bacterial cell. This was achieved in two steps. The first consisted of cleaving the KDO from R595 bacteria (class Re) by treating the cells with acetic acid at 100° for 90 min. In a second step, additional lipid A was attached onto the surface of these acid-treated cells in a manner analogous to that used for complexing lipid A to BSA.

These acid-treated cells enriched with lipid A on their surface were used to immunize rabbits. For comparison acid-treated cells without additional lipid A and non-treated killed bacteria were used. The various antigens were administered intravenously in a series of four injections at day 1, 4, 7 and 11 and blood was collected six days after the last injection.

Anti-lipid A activity was measured by the passive hemolysis of human red cells (HRC), coated with lipid A on alkali-treated lipid A.

Tab. 6 shows the antibody titres obtained by the three antigens.

	Whole bacteria	Acid-treated bacteria	Acid-treated bacteria and Lipid A
Hemolytic titres	1/16	1/128	1/1028

Tab. 6 : Anti-lipid A response to the three different bacteria preparations.

The anti-lipid A response to non-treated cells is very weak (1/16). It becomes higher after treating the cells with acid (1:128). By far the highest response (1:1028) is obtained by acid-treated cells coated with additional lipid A.

Specificity of anti-lipid A antibodies

The specificity of these antibodies for lipid A was demonstrated in absorption tests using lipid A-coated formalinized human red cells. Absorption of the antiserum with the above cells led to loss of the anti-lipid A activity. Part of the absorbed antibodies could be recovered by treating the cells with Glycine Buffer pH 2.5.

The specificity of anti-lipid A antibodies was also demonstrated by the specific lysis of lipid A-sensitized liposomes.

Opsonising activity of anti-lipid A antibodies

Anti-lipid A antibodies can interact with the lipid A part of a large number of serologically distinct lipopolysaccharides. As a result, opsonization of *E. coli* 0111 live bacteria with anti-lipid A antiserum leads to enhanced phagocytic clearance of these cells in the mouse peritoneum.

In blood clearance tests in the mouse using heat-killed *S. enteritidis* bacteria a low but significant enhancement of the clearance of these cells was obtained after incubating with anti-lipid A antiserum.

Occurrence of anti-lipid A antibodies and their possible biological significance

Apart from rabbits, immune titres of anti-lipid A antibodies were also produced in dogs and goats. They were also found to occur naturally in the sera of different animals. Anti-lipid A antibodies occur naturally in the sera of most humans. Screening of several hundred sera from healthy and sick adults and children, showed the presence of these antibodies in hemolytic titres ranging from 1:8 to 1:250.

The biological significance of anti-lipid A antibodies is not known. Their role for the organism may be a protective, damaging one, or both. Thus the interaction of these antibodies with lipid A, which is the toxic principle of lipopolysaccharides, may lead to detoxification. In this case anti-lipid A antibodies would act protectively and would play a role in endotoxin tolerance. On the other hand these antibodies may be playing a role in the various activities of endotoxin. It is well known that lipid A has a high affinity for cell membranes and tissues. It is thus possible that antibodies reacting with cell- or tissue-bound lipid A might lead to activation of humoral or cellular mechanisms, resulting in cell or tissue damage and the release of biologically active substances. This would mean that an immune mechanism associated with the lipid A is involved in endotoxic activity.

In any case, the knowledge that anti-lipid A antibodies really do exist introduces new factors in the field of endotoxin. As a result a remodelling of existing thoughts and ideas may be necessary in order to accommodate these factors.

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I

The Partial Acid Hydrolysis of Polysaccharides: A New Method for Obtaining Oligosaccharides in High Yield

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An apparatus is described for continually stepwise hydrolysis of polysaccharides. The oligosaccharides formed are separated by dialysis and collected on a charcoal-celite column. They are thus protected from further degradation and, at the same time, separated from monosaccharides formed. The period of each hydrolysis step, the length and temperature of dialysis, and the temperature of hydrolysis can all be independently varied. Moreover, any acid can be used, mineral acids or substituted soluble resins. As examples the results obtained with dextran and some bacterial polysaccharides are discussed.

The detailed molecular structure of homo- and heteropolysaccharides can generally be elucidated by partial acid hydrolysis with subsequent structural analysis of the resulting oligosaccharides. Although partial hydrolysis methods are clearly of crucial importance in such structural studies, most of the techniques described hitherto have limitations regarding the yields of oligosaccharides which are usually low because small fragments formed early in the process are unprotected and thus progressively degraded to monosaccharides as hydrolysis of the larger fragments proceeds. Methods have been described whereby oligosaccharides formed by enzymic hydrolysis, may be removed by continuous dialysis [1], and Painter has proposed the use of a non-dialysable soluble resin (polystyrene sulphonic acid) in a similar manner [2,2a]. An ideal way of protecting oligosaccharides from further degradation is to remove them from the hydrolysis mixture as soon as they are formed. This implies that a stepwise form of hydrolysis should be employed during which the polysaccharide is heated for a short time in acid solution and the oligosaccharides formed are subsequently removed by dialysis. This process is repeated continuously until all the polysaccharide has been converted to oligosaccharides.

The method described in this paper utilises the principle of stepwise hydrolysis and dialysis. The period of hydrolysis, the length of dialysis, and the temperature of hydrolysis can all be independently varied. Moreover any acid can be used, mineral acids or substituted soluble resin. The results show that a high yield of oligosaccharide can be expected if the conditions are carefully chosen to suit the properties of the polymer. A simpler version of the apparatus has been briefly described recently [3].

Description of the Apparatus

A diagram of the apparatus is shown in Fig. 1. (A) is the heating chamber usually maintained at 100°; (B) is the cooling chamber usually maintained at room temperature; and (C) is the dialysis chamber.

A peristaltic pump (P) circulates the polysaccharide in acid solution in the direction shown by the arrows. The polysaccharide is thus hydrolysed in

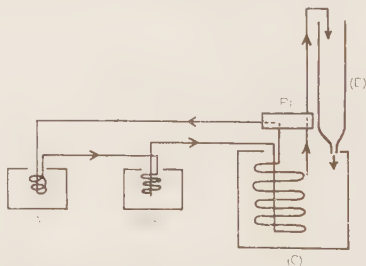


Fig. 1. Scheme of the continuous hydrolysis apparatus. (A) heating chamber; (B) cooling chamber; (C) dialysis vessel; (D) charcoal-celite column; (P) pump

chamber A and the products cooled and dialysed in chambers B and C respectively. Undegraded polysaccharide is returned to A for reprocessing.

Via a second circuit effected by the same pump (P), the oligosaccharides in the outer dialysate are fed as fast as possible from C onto a charcoal-celite column [D, 5 cm in diameter, 50 g charcoal—celite (1:1)] maintained at 2° by a cooling jacket. The oligosaccharides are thus protected from back dialysis

and subsequent further hydrolysis (*cf.* [9]). They are adsorbed on the column and the oligosaccharide-free solution, which may contain monosaccharides, returns to the dialysis chamber.

Heating Chamber (A). This consists of a glass coil (inner volume 1–10 ml) immersed in a heating bath. The internal diameter of the coil should be small (about 2 mm) to provide a regular forward flow of the solution. The volume of the coil and the rate of pumping determine the length of each hydrolysis step.

Cooling Chamber (B). This consists of a glass coil 40 to 50 cm long (volume 7.0 ml) immersed in 1 l of water at room temperature. This coil cools the hot solution from A before it enters C.

Dialysis Vessel (C). This is a glass jar which contains the dialysis tube holder and the outer dialysate. When mineral acid is used for hydrolysis, the outer dialysate contains the same acid in the same concentration. If soluble substituted resin is used, the outer dialysate contains distilled water only. Dialysis is carried out by passing the cooled hydrolysate through a dialysis tube 6.5 m long. With this length a dialysis period of 40 min can be conveniently adjusted, which proved to be suitable for obtaining di- to hexa-saccharides in optimal yields. This tubing is mounted on a plastic tube holder and is completely immersed in the outer dialysate.

Plastic Dialysis Tube Holder. This can be made from any rigid acid-resistant plastic. It consists of two circular plates 13.0 cm in diameter and 0.6 cm thick (Fig. 2A and B) joined through a central stem 10 cm long and 7.0 cm in diameter (Fig. 2D). It is important to have this stem so thick in order to keep the volume of liquid necessary to cover the tubing to a minimum. Each plate has holes (diameter 0.6 cm) arranged in two concentric circles. The outer circle has sixteen such holes and the inner twelve. These holes provide attachment for twenty-eight glass rods (11.2 cm long) on which the dialysis tubing is mounted (Fig. 3A).

The top plate (Fig. 2A) has two extra large holes to permit the entry and exit of the ends of the dialysis tube, and several recesses in its circumference so that a thermometer can be inserted into the chamber. The lower plate (Fig. 2B) has a serrated edge to allow free circulation of the outer dialysate from the stirrer to the upper part of the chamber. Efficient stirring can be effected by a magnet enclosed in a plastic holder as shown in Fig. 3B. A cylindrical rod (Fig. 3C) at the middle of the magnet fits into a hole at the centre of the lower plate (Fig. 3D) and thus keeps the magnet in position during stirring. Three short legs (Fig. 3H) keep the tube holder a few millimetres above the magnet so that the latter can rotate freely under the lower plate.

To mount the dialysis tube on the holder, the rods of the inner circle are first fitted and the dialysis tube

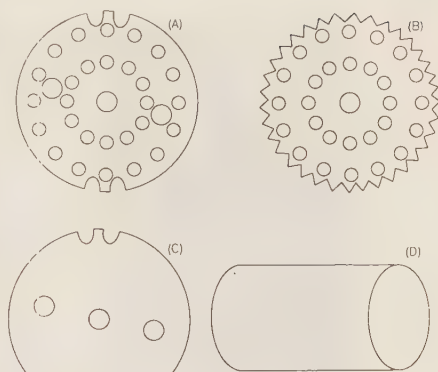


Fig. 2. Parts of the dialysis tube holder. (A), (B), and (C) circular plates; (D) central stem

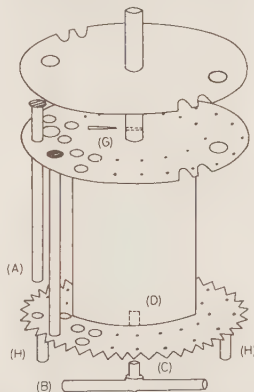


Fig. 3. Dialysis tube holder, partly assembled (A) two of the 28 glass rods; (B) magnetic stirrer with (C) cylindrical rod; (H) two of the three legs; (G) plastic pin

is wound spirally downwards. When the inner circle is complete, the rods of the outer circle are inserted and the winding of the tube continued spirally upwards around these rods. A third plate (Fig. 2C) is now placed on top of the rods to keep them in position. This plate is held in position by a plastic pin (Fig. 3G). The dialysis vessel should be just wide enough to permit entry of the holder so that the volume of the outer dialysate can be kept to a

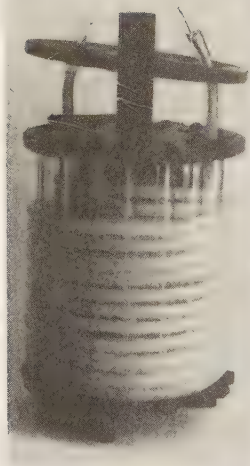


Fig. 4. Photograph of the assembled dialysis tube holder carrying the dialysis tubing

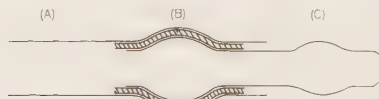


Fig. 5. Connection of the ends of the dialysis tube to a glass tubing. (A) end of the dialysis tube; (B) silicon tubing; (C) glass tubing

minimum. A photograph of the complete holder with tubing is shown in Fig. 4.

Dialysis Coil. This consists of Visking tubing 6.5 m long and 6.5 mm in diameter inside which silicon tubing 5 mm in diameter is inserted. The silicon tubing provides a rigid support for the dialysis tubing which would otherwise collapse, and at the same time reduces the capacity of the dialysis tube so that the dialysing surface per ml of liquid is larger. With both silicon and dialysis tubes kept wet, the silicon tube is inserted by the aid of a glass rod to which it is fixed. One should always ensure that the dialysis tubing is evenly distributed over the length of the silicon tube as corrugation will impede the smooth flow of the dialysing solution. The silicon tube is then cut short 5 cm from each end of the dialysis tube and sealed at both ends with small glass rods. Fig. 5 shows how the ends of the dialysis tube (A) are connected to the rest of the circuit. A

piece of silicon tubing 2 cm long (B) and of about the same diameter as the dialysis tube (A) is inserted into the latter and a glass connector (C) with blown out ends pushed through the silicon tubing to make a permanent connection.

The capacity of the dialysis tubing should now be about 40 ml and the total circulating volume about 60 ml. In use the dialysis coil should always be immersed in the acid solution and never allowed to dry out with acid on its surface. After each experiment, it should be thoroughly washed by pumping water through and by immersing it repeatedly in fresh lots of water. It should be stored on its holder in distilled water in the cold. By using 0.5 N acid for hydrolysis the dialysis tube could be used for at least a year. If stronger acid solutions are used, the dialysis chamber should also be cooled.

EXPERIMENTAL PROCEDURE AND RESULTS

Partial Hydrolysis of Dextran

The conditions used for hydrolysis are summarised in the Table. The apparatus in this experiment was similar to the one described in this paper but with a longer dialysis tubing. The rather long dialysis period favoured diffusion of high molecular weight material. Furthermore no charcoal-celite column was used in this experiment. The outer dialysate was simply changed once during the experiment. After 34 cycles the experiment was discontinued, although about half of the polysaccharide, which had not been degraded to a dialysable size, was still in the circuit. The pooled dialysates were neutralised with $\text{Ba}(\text{OH})_2$ and after centrifugation the supernatant was freeze-dried. Chromatography was carried out on thin-layer plates in butanol-pyridine-water (6:5:5, by vol.). This solvent gives a very good separation of higher oligosaccharides. All oligosaccharides up to isomaltooctaose separated (see Fig. 6). Still higher oligosaccharides were also present, streaking down to the origin. Analysis was carried out with respect to free and total glucose. Free glucose was determined by the glucose oxidase test and total glucose by the same method after hydrolysis in 1 N H_2SO_4 at 100° for 4 hours.

Free glucose accounted for 4.7% of the dialysable product. Prolongation of the hydrolysis is not expected to change substantially the percentage of free glucose. As seen from the Table about 4 g of oligosaccharides were obtained after hydrolysis of about half of the original dextran, which would correspond to a total yield of about 90% oligosaccharides if hydrolysis had been prolonged. When dextran is hydrolyzed by conventional free solution methods, 30 to 40% of free glucose [4] and 50% of oligosaccharides (0.5 N H_2SO_4 , 3 h, 100°), are obtained.

Table. *Partial acid hydrolysis of dextran and of four bacterial polysaccharides*
(Conditions used and yields of oligosaccharides obtained)

	Dextran	Polysaccharide derived from:			
		<i>S. minnesota</i> S form	<i>S. minnesota</i> R 60	<i>S. typhimurium</i> TV 149	<i>E. coli</i> EH 100
Weight of polysaccharide (g)	8	2	0.7	1.5	0.88
Volume of hydrolysis mixture (ml)	300	55	50	50	50
Acid strength (H ₂ SO ₄)	0.5 N	0.5 N	0.5 N	0.5 N	1.0 N
Rate of pumping (ml per min)	3	1	1	1	4
—Time needed for one cycle (min)	100	55	50	50	13
Volume of heating coil (ml, 100°)	8.5	1.1	1.0	1.0	12
—Length of each heating step (min)	2.8	1.1	1.0	1.0	3
Volume of dialysis coil (ml)	210	40	40	40	40
—Length of each dialysis step (min)	70	40	40	40	10
Volume of outer dialysate (ml)	2,500	800	800	800	800
Temperature of outer dialysate	RT	RT	RT	RT	9°
Weight of charcoal-celite mixture (g)	—	50	50	50	50
Temperature of charcoal-celite	—	2°	22°	2°	2°
Total number of cycles	34	30	36	30	74
Yield of oligosaccharide fraction (g)	4.0	0.43	0.22	0.48	0.315

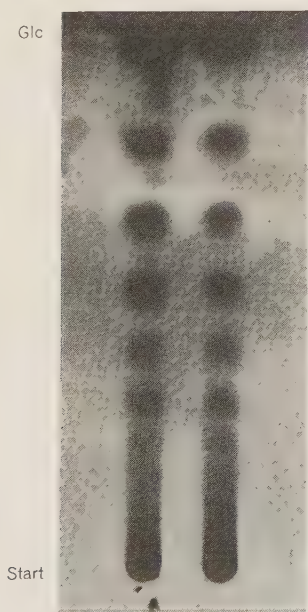
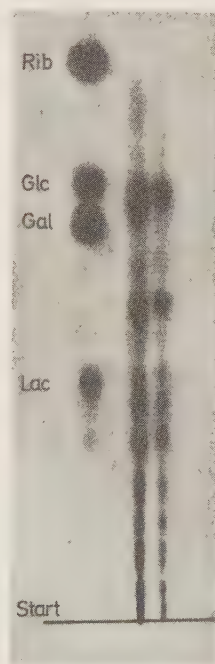


Fig. 6. Thin layer chromatogram of the hydrolysate of dextran, (two different concentrations), with glucose (Glc) as the fastest spot and the oligosaccharides of the isomaltose series as the slower components

Fig. 7. Paper chromatogram of the neutral fraction of the hydrolysate of *Salmonella minnesota* S form polysaccharide (two different concentrations). Reference sugars (left side) are lactose (Lac), galactose (Gal), glucose (Glc) and ribose (Rib)

Partial Hydrolysis of Bacterial Polysaccharides

The lipopolysaccharides, obtained from four bacterial strains by extraction with phenol—water [5], were treated with acetic acid (0.1 N, 100°, 3 h) to remove the lipid A component [6]. The water soluble polysaccharides obtained were freeze-dried, weighed and partially hydrolysed under conditions summarized in the Table. In all cases the charcoal-celite column was kept at 2° throughout the experiment. The acid and monosaccharides were removed by washing the column with distilled water. Desorption of the oligosaccharides was carried out by washing the charcoal column with 30% aqueous ethanol, at room temperature or at 30°.

About 22% of hydrolysed *Salmonella minnesota* S. form polysaccharide was recovered in the form of neutral oligosaccharides, ranging from di- to hexasaccharides [3,7]. Fig. 7 shows a paper chromatogram of the neutral oligosaccharides in butanol—pyridine—water (6:4:3, by vol.). It can be seen that monosaccharides are completely absent.

Mainly acidic oligosaccharides in about 30% yield (Table) were obtained from the polysaccharides derived from the R mutants *Salmonella minnesota* R60 [3] and *Salmonella typhimurium* TV149 [8]. When *S. minnesota* R60 was hydrolyzed under optimal conventional conditions (1 N H₂SO₄, 30 min, 100°) the yield of oligosaccharides was in the order of 2% of the starting polysaccharide [10].

An R mutant of *E. coli* (*E. coli* EH100 supplied by H. Mäkelä, States Serum Institute, Helsinki, Finland) was hydrolyzed under different conditions which included stronger acid and larger heating steps (see the Table). In this case the proportion of neutral to acidic oligosaccharides was higher than in

the previous experiments. The yield of oligosaccharides was 35% of the starting material (Table). The yield of oligosaccharides obtained after free solution hydrolysis (1 N H₂SO₄, 30 min, 100°) amounted to 3% of the starting polysaccharide.

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A New Method for the Extraction of R Lipopolysaccharides

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A method is described which is specific for the extraction of lipopolysaccharides from R form bacteria. The extraction mixture which is monophasic, consists of aqueous phenol, chloroform and petroleum ether. R form lipopolysaccharides (glycolipids), due to their lipophilic nature, are completely soluble in the mixture. S and T form lipopolysaccharides as well as proteins, nucleic acids, and polysaccharides, are insoluble, and they are excluded from the extracts. The method is mild, as it can be carried out at below 10°. The yields are generally higher than those obtained by phenol-water extraction, and the products are usually water-soluble. Lipopolysaccharides have been successfully extracted from all R form bacteria so far attempted.

Many methods have been developed for the extraction of endotoxic lipopolysaccharides from *Salmonella* and other gram-negative bacteria [1,2]. One of the methods, which is widely used, is the phenol-water procedure [3,4], because it is applicable to many different groups of bacteria and because it is one of the few methods by which also lipopolysaccharides from R mutant bacteria can be extracted. In this method, dried bacteria are treated at 69° in a mixture of phenol and water (45:55, v/v). On cooling, the mixture, which is monophasic above 68°, separates into a phenol phase containing mainly proteins, and a water phase containing lipopolysaccharide and nucleic acid.

However, there are recent reports in the literature describing unexpected results. Ashwell *et al.* [5] extracted *Xanthomonas campestris* bacteria with phenol–water and obtained a phenol-soluble lipopolysaccharide. Similarly, Raff and Wheat [6] applied the phenol–water procedure to *Citrobacter* strains and obtained the respective lipopolysaccharides from the phenol phase. Finally, Kasai and Nowotny [7], who investigated a *Salmonella* R mutant, isolated the corresponding heptose-less lipopolysaccharide (glycolipid) from the phenol interphase, though, in our hands, the phenol–water extraction of the same mutant yielded an identical lipopolysaccharide (glycolipid) in the water phase [8], however, in a rather low yield.

All the lipopolysaccharides found to be soluble in the phenol phase seem to have the common characteristic of being hydrophobic in nature. The lipopolysaccharides from *X. campestris* and *Citrobacter* contain 3-*N*-acetylramino-3,6-dideoxyhexose [5,9,10] and the heptose-less lipopolysaccharide of the R mutant contains besides 2-keto-3-deoxyoctonate (KDO) about 70% of lipid A [8].

Unusual Abbreviation. KDO, 2-keto-3-deoxyoctonate.

The present paper describes an extraction method which is specific for R form lipopolysaccharides which are obtained as water-soluble preparations of high purity regarding contamination with proteins and nucleic acid. S form lipopolysaccharides are excluded from the extract, and also certain polymers, like glucans, are not co-extracted.

MATERIALS AND METHODS

Bacteria

The following S and R strains were used: *S. minnesota* S form, mR345, mRz, mR595 [8], *S. godesberg* gR15 (isolated by J. Schlosshardt), and *S. typhimurium* TV 161 [11]. They were cultivated as described previously [12]. After harvesting, the bacteria were washed with distilled water. Saline or any other salt solution should be strictly avoided, as such a treatment may result in a very poor lipopolysaccharide yield. The washed bacteria are treated successively with ethanol, acetone and twice with ether. They are dried *in vacuo* over CaCl₂ to constant weight.

Extraction Mixture

A mixture containing liquid phenol (90 g dry phenol + 11 ml water), chloroform and petroleum ether (b. p. 40–60°) in a volume ratio of 2:5:8, respectively, was used. This mixture was a monophasic system if the phenol used was dry. If water was present in the original phenol preparation, the mixture was cloudy; it could be made clear by adding solid phenol.

Extraction Procedure

The dried bacteria (50 g) were placed in a centrifuge vessel and the extraction mixture (200 ml)

was added. The suspension was then homogenised with the Ultra-Turrax homogenisator (Janke & Kunkel) for 2 min with cooling so that the temperature remained between 5° and 20°. This treatment was not meant to break the bacteria but to obtain them in a fine suspension. If the bacteria were already in a fine form, stirring of the mixture for a few minutes was sufficient. Sometimes the suspension after homogenising was very viscous. In this case more extraction mixture was added. The bacteria were then centrifuged off (5000 rev./min, 15 min) and the supernatant which contained the lipopolysaccharide was filtered through filter paper into a round flask (1 l). The bacterial residue was extracted once more with the same amount of extraction mixture, stirred and centrifuged as above and the supernatant was added to the first extract. The extraction could be repeated for a third time.

The pooled supernatant solutions had a light yellow to dark brown colour. Petroleum ether and chloroform were then removed completely on a rotary evaporator at 30–40° (or in high vacuum at below 0°). Should the remaining phenol now crystallise, sufficient water was added to dissolve it. The solution was transferred into a glass centrifuge pot and water added dropwise until the lipopolysaccharide precipitated. Addition of water was stopped when the lipopolysaccharide started settling down after the mixture was allowed to stand for 1 to 2 min. Although precipitation of the lipopolysaccharide is complete long before the phenol is saturated with water, care must be taken not to add too much water as this causes formation of two phases. The precipitated lipopolysaccharide was then centrifuged (3000 rev./min, 10 min), the supernatant decanted, and the tube allowed to stand for 2 to 3 min upside down. It was then wiped inside with filter paper. The precipitate was washed two to three times with small portions of 80% phenol (about 5 ml) and the inside of the tube was wiped with filter paper after decantation of the supernatant. Finally, the precipitate was washed three times with ether to remove any remaining phenol, and dried *in vacuo*. The lipopolysaccharide was taken up in distilled water (50 ml), warmed to about 45°, and vacuum was carefully applied to remove the air. It was then shaken for a few minutes whereby a viscous, sometimes very viscous, solution was obtained. The viscosity may be reduced by placing the solution in an ultra vibrator for 5 min. The lipopolysaccharide solution was centrifuged once at high speed (100000 × g, 4 h). The resulting sediment was clear and transparent, so that it was sometimes difficult to recognise until the supernatant was decanted. The lipopolysaccharide was redissolved in water and freeze-dried.

The supernatant solution after ultracentrifugation contained negligible amounts of material and was discarded. It did not contain O-specific hapten [13].

RESULTS

Extraction of S and T₁ Form Bacteria

A number of S and T₁ lipopolysaccharides which had been extracted with phenol–water [3, 4, 8], when tested for their solubility in the phenol–chloroform–petroleum ether mixture, were found to be insoluble. When *S. typhimurium* S form and *S. friedenau* T₁ form bacteria were subjected to the new extraction method no material was found in the extract. Phenol–water, on the other hand, extracted from the same batches of bacteria the respective lipopolysaccharides in usual yields.

Extraction of R Form Bacteria and Analysis of the Respective Lipopolysaccharides

The new method of extraction with a mixture of phenol, chloroform and petroleum ether, as described in Methods, has been successfully applied to a large number of R mutant bacteria and, without exception, the respective lipopolysaccharides were obtained. In order to compare lipopolysaccharides extracted by phenol–water [3, 4] with those obtained by the new method, one batch of each of 5 bacterial R mutants strains was split and each half was extracted separately by the two procedures respectively. The Table summarizes the results.

Table. Comparative analysis of R lipopolysaccharides extracted by the phenol–chloroform–petroleum ether and phenol–water methods

A = values for phenol–chloroform–petroleum ether extracts.
B = values for phenol–water extracts. NT = Not tested.
— = Not tested because of poor yield

Lipopolysaccharide		Yield	KDO	Heptose	Glucose	Galactose	Lipid A
		%	%	%	%	%	%
<i>S. minnesota</i> RZ	A	2.4	14.4	17.7	0	0	64
	B	0.88	15.0	15.4	0	0.5	52
<i>S. minnesota</i> R595	A	5.5	23.0	0	0	0	70
	B	0.6	21.0	0	0	0	65
<i>S. godesberg</i> R15	A	5.3	17.0	0	0	0	68
	B	0.1	—	—	—	—	—
<i>S. minnesota</i> R345	A	1.2	15.0	17.5	4.5	8.5	NT
	B	NT	12.0	14.0	4.0	9.0	NT
<i>S. typhimurium</i> TV 161	A	3.2	10.8	16.0	4.0	7.7	NT
	B	2.0	8.9	15.8	6.8	8.7	NT

In each case the yield obtained by the new method was significantly higher. From *S. godesberg* gR15 practically no lipopolysaccharide was obtained with phenol–water, but more than 5% by the new method (see the Table). Sugar analyses performed on the respective pairs of lipopolysaccharides showed that they contained the same sugar constituents, in about the same quantities.

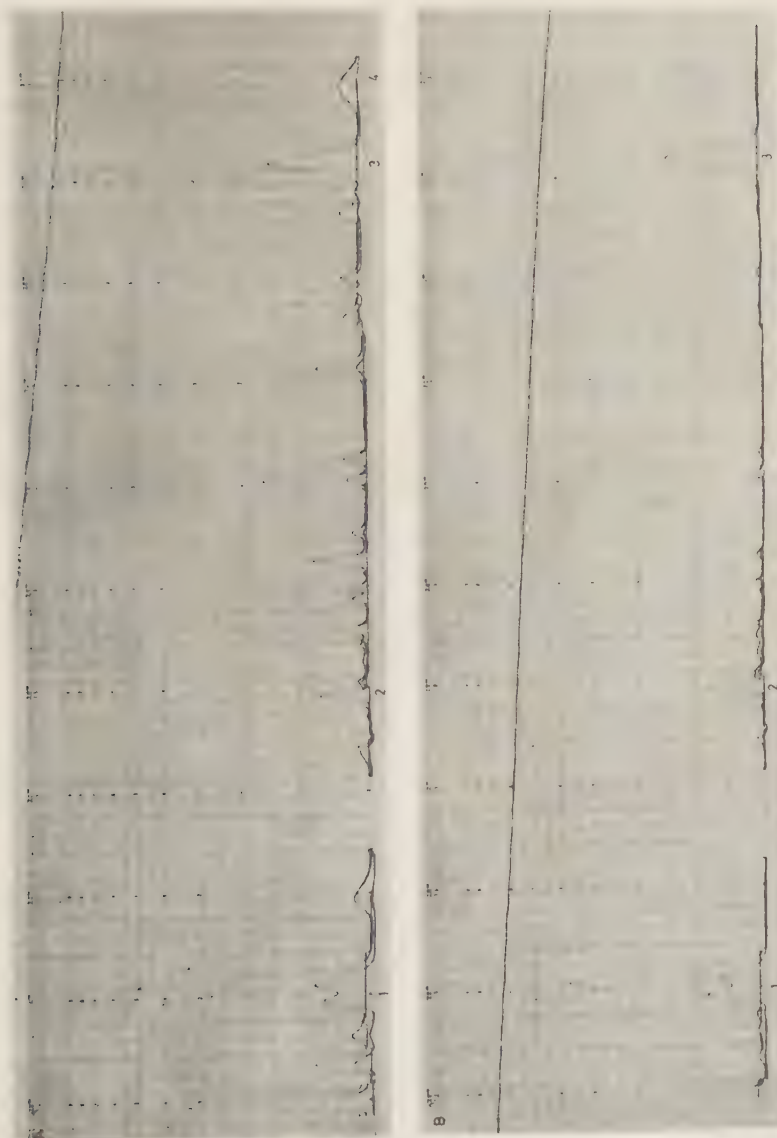


Fig. 1. Amxro acid spectra of the hypophosphoribosides of *S. minusota* m/z obtained by the phenol water method (1) and by the phenol chloroform petroleum ether method (2). 1 ethanodamine, 2 ethanodamine phosphate, 3 glucosamine phosphate, 4 celvioxamine.

Nucleic acid was absent from all lipopolysaccharides. The protein (or peptide) content in each of the lipopolysaccharides obtained from the mutant mRz by the respective procedure was compared in the amino acid analyzer after hydrolysis of the extracts in 6 N HCl for 24 h (Fig. 1A and 1B). A number of amino acid peaks are seen on Fig. 1A (phenol—water extraction), corresponding to a 0.1 to 1% total in the preparation. This amount is significantly reduced in the phenol—chloroform—petroleum ether extracted lipopolysaccharide (Fig. 1B). Peaks of ethanolamine and glucosamine are seen in both lipopolysaccharides. The phenol—water extracted preparations shows the presence of small amounts of galactosamine, indicating contamination with S form lipopolysaccharide which is synthesized by this mutant mRz due to a leaky defect [14]. This S form contaminant is absent from the phenol—chloroform—petroleum ether extracted preparation, a conclusion derived from the fact that the galactosamine peak is absent. This is also supported by the absence of galactose.

Sequential Extraction of S. minnesota mR60 Bacteria

On extraction of *S. minnesota* mR60 bacteria with the phenol—chloroform—petroleum ether mixture, a lipopolysaccharide (1% yield) was obtained. Sugar analyses revealed the presence of KDO (12%), heptose (17%), glucose (6%) and galactose (6%). No ribose was detectable. The bacterial residues of the first extraction were subsequently extracted with phenol—water and a second lipopolysaccharide was obtained (0.7% yield) containing KDO (9%), heptose (13%), glucose (7%), galactose (10%), and in addition, ribose (10%). It is concluded, that the mutant mR60 synthesizes two lipopolysaccharides, one being a typical R lipopolysaccharide, the other being a T_1 -like lipopolysaccharide (see also [8]).

Isolation of O-Specific Hapten

When cells of *S. typhimurium* TV161 are extracted with phenol—water and the extract is subjected to high speed centrifugation, the corresponding R lipopolysaccharide is obtained as a sediment (2% yield, see Table 1), while the supernatant solution contains O-specific hapten [13].

Extraction of the same cells by the phenol—chloroform—petroleum ether method yields R lipopolysaccharide (3.2% yield, Table). No O-specific hapten is found in the extract. The cell residue after this extraction was treated with phenol—water. The water-phase obtained yielded, on high speed centrifugation, a small amount of sediment (0.4%) and a supernatant containing the O-specific hapten. Its presence was demonstrated after hydrolysis by paper chromatography: glucose, galactose, mannose, rhamnose and abequose were detected [13].

DISCUSSION

An important factor in the phenol—water method for the extraction of bacteria is the distribution of lipopolysaccharide between (water-saturated) phenol and (phenol-saturated) water. Because of their relative hydrophilic character, lipopolysaccharides from enterobacterial S (wild type) forms are generally excluded from the phenol phase and they are found almost exclusively in the water phase. However, in the case of more lipophilic lipopolysaccharides, such as those derived from R mutants, a different distribution between the two phases could be expected, and here, several, otherwise not so critical factors could govern the yield, in which such lipopolysaccharides are obtained from the water or phenol phase, respectively. One could imagine, for instance, that the kind and/or quantity of cations, or small differences in the temperature to which the phenol—water mixture is cooled, might influence the distribution of the more lipophilic lipopolysaccharides, and it is not surprising on the basis of these considerations that reports have been published where a lipopolysaccharide was found, after extraction, in the water phase, in the phenol phase or at the interphase ([7], see also [5, 6]) of a phenol—water system. The lipopolysaccharide in question was derived from a *Salmonella* R mutant and is known to be highly lipophilic since it contains only KDO and about 70% of lipid A [8]. The same is true for R mutant lipopolysaccharides in general, which are devoid of the long O-specific side chains which confer hydrophilic properties to the S form lipopolysaccharides. Regarding the S form lipopolysaccharides which have been isolated from the phenol or interphase [5, 6] it is assumed that they are relatively lipophilic due to the presence of deoxy- and deoxy-N-acetyl amino-hexoses.

The present paper describes an extraction method, where aqueous phenol, chloroform, and petroleum ether is used as extraction mixture, which has been applied successfully to *Salmonella* R mutants of all R classes (Ra to Re [8]) for the isolation of the respective R lipopolysaccharides. No S or T_1 lipopolysaccharides are extracted from either S or T_1 form bacteria, respectively.

The R lipopolysaccharides which were obtained by the new method in higher yields, compared with the phenol—water method, proved to be pure with respect to contaminants such as protein, nucleic acids and polymers like glucan. The S form lipopolysaccharide present in a leaky mutant (mRz) was completely excluded from the new extract. Also, the O-specific hapten present in Rb class mutants is not co-extracted with the Rb lipopolysaccharide; it can be obtained when the cell residues are subsequently extracted with phenol—water. When cells of the R mutant strain of *S. minnesota* mR60 [8] were extracted with phenol—chloroform—petroleum ether, a R lipopolysaccharide was obtained. A second (T_1 -like) lipo-

polysaccharide [8] containing ribose could be extracted by further extraction of the cell residues with phenol—water.

The new method of extraction can be applied to small amounts of bacteria. It is relatively mild, because it is carried out at temperatures between 5° and 20°. It is also time-saving, because the long dialysing periods and the three-fold ultra-centrifugation of the phenol—water method are not necessary in this procedure. The R lipopolysaccharides obtained are usually completely soluble in water. Addition of salt, such as $MgCl_2$ or $CaCl_2$ (or larger amounts of NaCl) precipitates these lipopolysaccharides from their solution. This may be a reason why lipopolysaccharides are not extractable from bacteria that had been washed with salt solutions during harvesting.

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Lipid A: Chemical Structure and Biological Activity

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Further details of the chemical structure of lipid A of *Salmonella* have been evaluated. It was found that pyrophosphate bridges interlink β -1,6-linked glucosamine disaccharide units, which are esterified by lauric, palmitic, and 3-D-myristoylmyristic acid and which are substituted at the amino groups by 3-D-hydroxymyristic acid. Lipid A is the biologically active component of lipopolysaccharides. Free lipid A, solubilized by complexing with bovine serum albumin, exhibits strong endotoxic activity in a number of biological tests. Lipid A exhibits immunogenic properties when suitably exposed on the bacterial surface. Immunization of rabbits with lipid A leads to the production of specific antibodies to lipid A that are capable of cross-reacting with a large variety of lipopolysaccharides.

Lipopolysaccharides are located in the cell wall of gram-negative bacteria where they form, complexed with lipids and proteins, the outer membrane of the cell. They represent the O antigens and the endotoxins of these organisms (for reviews see [1]). Lipopolysaccharides consist of three regions of contrasting chemical and biological properties. The O-specific polysaccharide (region I), carrying the main serologic specificity, is linked to the core polysaccharide (region II), which is common to groups of bacteria. The core is linked through a 2-keto-3-deoxyoctonate (KDO) trisaccharide to the lipid component (region III) termed lipid A [2]. Biochemical and genetic investigations on endotoxins performed in the last decades have led to an understanding of the detailed structure, the biosynthesis, and the underlying genetics of the polysaccharide component of salmonella lipopolysaccharides. These results were obtained by the use of so-called rough (R) mutants that are defective in the synthesis of the complete lipopolysaccharide. Thus, mutants were isolated from strains of *Salmonella*, *Escherichia coli*, *Shigella flexneri*, and other bacteria whose lipopolysaccharides lack the O-specific chains or additional parts of the core. However, knowledge about the chemistry of the lipid A component of lipopolysaccha-

rides was rather fragmentary until recent efforts in various laboratories led to elucidation of the structure and biosynthesis of this part of endotoxins. Simultaneously, biological studies on lipid A were resumed; these had led 15 years ago to strong indications that lipid A played a role in endotoxicity. This concept, however, was not generally accepted at that time [3].

The present paper summarizes recent results obtained in our laboratory on the chemistry and biology of lipid A. Most studies were performed on *Salmonella*, but comparison will be made with other bacterial groups.

Chemical Structure of Lipid A

Isolation of lipid A. In principle there are two sources of lipid A. A number of mutant strains are known that are devoid of heptose (Hep) and therefore synthesize a defective lipopolysaccharide containing only lipid A and KDO. These natural products, generally designated as glycolipids, proved to represent good starting materials for investigations on lipid A [4].

Alternatively, lipid A may be isolated from any lipopolysaccharide by hydrolysis with mild acid [5, 6]. This treatment cleaves the ketosidic linkage of KDO to lipid A. In this way water-insoluble preparations of lipid A are obtained. Due to the acidic conditions of isolation, these preparations may be degraded to some extent. In the case of

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lipopolysaccharides containing more stable linkages to lipid A, more drastic conditions of hydrolysis are necessary, and some destruction is inevitable (G. Rosenfelder, unpublished observations).

Lipopolysaccharides are generally extracted in our laboratory with phenol and water (in the case of smooth (S) forms [7]) and with a phenol-chloroform-petroleum ether mixture (in the case of R forms [8]). After ultracentrifugation, the preparations may be purified further by electro-dialysis (C. Galanos, unpublished observations). Metal ions and amines like ethanolamine (EtN), spermidine, and putrescine, which contaminate lipopolysaccharides, are thus removed. With some S and R lipopolysaccharides, this treatment leads to their deposition as an insoluble gel on the anode membrane, from which they can be collected. The material can then be solubilized by conversion to the sodium salt.

Structure of the lipid A backbone. It is known that salmonella lipid A contains glucosamine, phosphate, and long-chain fatty acids [5, 9]. Ethanolamine, which is present in the core polysaccharide (where it is linked to heptose through a pyrophosphate and to KDO through a phosphate bridge), also occurs as a constituent in the lipid A region linked in a still unknown way.

Regarding the structure of lipid A, two proposals were made that, at first sight, seemed to exclude each other. Nowotny [10] concluded from his findings that glucosamine residues in lipid A were linked together by 1, 4 bridges of phosphate diester groups. On the other hand, results of Burton and Carter [11] suggested the existence of glycosidic linkages between glucosamine (GlcN) units. As will be seen below, both kinds of linkages probably occur in lipid A.

Most of the more recent structural studies were performed on the glycolipids of Re mutants of *Salmonella* containing KDO and lipid A or of Rd₂ mutants containing additional heptose. Removal of the long-chain fatty acids by hydrazinolysis led to the recognition of the backbone structure of lipid A units [4, 12]. Pentasaccharides of the formula (KDO)₃-2,3-GlcN-β1,6-GlcN, phosphorylated in position 4' and/or 1, were isolated. These oligosaccharides could be converted by mild hydrolysis to the corresponding KDO-free phosphorylated glucosamine disaccharides.

Studies on model substances like RNA, cephalin, uridine diphosphate-N-acetyl glucosamine

(UDP-GlcNAc), or Hep-Hep-(P-P-EtN)KDO revealed that, under the conditions used, treatment with hydrazine for 10 hr led to a cleavage of pyrophosphate bridges, while phosphate bridges were attacked much more slowly. Hydrazinolysis was therefore performed under milder conditions (2 hr) with the glycolipid of an Rd₂ mutant (V. Lehmann, unpublished observations). After chromatography of the hydrazinolysate on Sephadex, three fractions (A-C) were isolated. Their structures were identified by application of hydrolysis with mild acid, treatment with phosphatase, and deamination with nitrous acid. Fraction A (figure 1) consisted of phosphorylated oligosaccharides corresponding to those mentioned above. Fraction B, which contained heptose, KDO, glucosamine, and phosphate in a ratio of 2:6:4:3 (or 4), could be identified as a dimer of fraction A interlinked by a pyrophosphate bridge. Fraction C, having the same molar ratio of constituents as B, represents an oligomer of several repeating units. In addition, fraction C contains 1 mole of amide-linked 3-hydroxymyristic acid per 2 moles of glucosamine.

These studies also confirmed our previous findings [13] that the three residues of KDO detec-

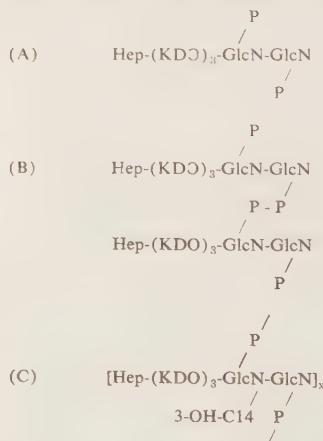


Figure 1. Three fragments isolated after limited hydrazinolysis of the glycolipid of a *Salmonella minnesota* Rd₂ mutant. Key to abbreviations used in figures: Hep = heptose; KDO = 2-keto-3-deoxyoctonate; GlcN = glucosamine; P = phosphate; EtN = ethanolamine; 3-OH-C14 = 3-D-hydroxymyristoyl.

table in glycolipids form a trisaccharide. When fraction A (or the corresponding heptoseless pentasaccharide from a *Re* mutant) was successively treated with nitrous acid (deamination, formation of substituted anhydromannose), and alkali (β -elimination of the oligosaccharide from position 3'), the oligosaccharides Hep-(KDO)₃ and (KDO)₃, respectively, could be isolated and identified after reduction with NaBH₄ (V. Lehmann, unpublished observations, see figure 2). It is the

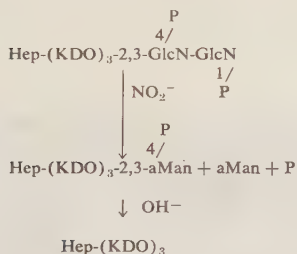


Figure 2. Isolation of a Hep-(KDO)₃ oligosaccharide from a *Salmonella minnesota* Rd₂ glycolipid. Similarly, a KDO trisaccharide was isolated from a *Re* glycolipid. (aMan = 2,5-anhydromannose.)

first time that KDO oligosaccharides were isolated from lipopolysaccharides.

Recently, Adams and Singh have shown that, in the lipopolysaccharide of *Serratia marcescens*, the lipid A backbone also contains β 1,6-linked glucosamine units [14, 15], while in *E. coli* 086 and *S. flexneri*, glucosamine oligomers with β 1,4 linkages are present [16].

Nature and linkages of fatty acids. Lauric, myristic, palmitic, and 3-D-(—) hydroxymyristic acid represent the long-chain fatty-acid constituents in lipid A of *Salmonella*. These fatty acids are released on acid hydrolysis, while treatment with alkali liberates, in addition, Δ^2 -tetradecenoic acid (α,β -unsaturated myristic acid [17]). It was concluded that this unsaturated acid was an artifact derived by β -elimination from O-acylated 3-hydroxymyristic acid ester. The substituent of the hydroxy fatty acid was then identified as myristic acid. Finally, it could be shown that after treatment of the glycolipid with mild alkali, 3-myristoxymyristic acid was liberated.

These findings, and the fact that the amino groups of the glucosamine units are substituted by 3-hydroxymyristic acid residues, led to the formula of a lipid A unit represented in figure 3 [17].

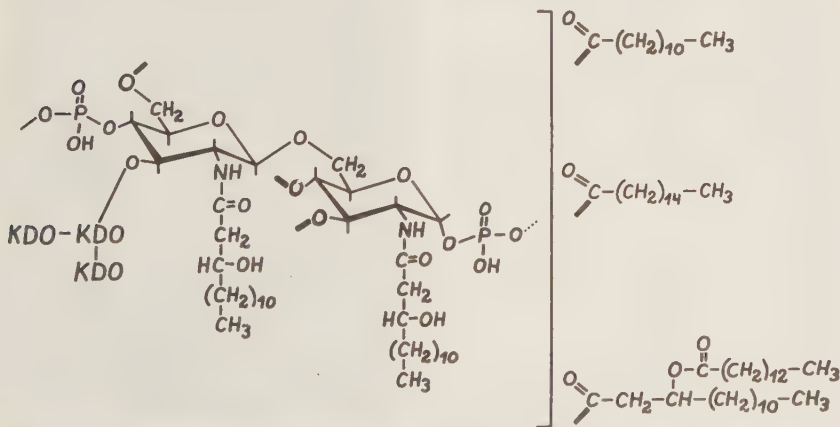


Figure 3. Proposed structure of a lipid A unit of the *Salmonella minnesota* R595 glycolipid with an attached KDO trisaccharide. The three fatty-acid residues shown are linked in an unknown distribution to the hydroxyl groups of the glucosamine residues available at positions 3, 4, and 6'. Additional small amounts of myristic acid and unsubstituted 3-hydroxymyristic acid may occur as esters linked to glucosamine.

The three available hydroxyl groups in positions 6', 3, and 4 of the glucosamine disaccharides are esterified by about equal amounts of lauric, palmitic, and 3-myristoxymyristic acid (and small amounts of myristic and 3-hydroxymyristic acid). The amino groups are substituted exclusively with 3-hydroxymyristic acid residues, whose hydroxyl group is free.

The investigation of lipopolysaccharides of genera other than *Salmonella* clearly revealed that the nature and distribution of fatty acids may vary [18]. Thus, different or no hydroxy fatty acids may be present in the lipid A structure from other bacterial groups (table 1).

It is well known that the removal of ester-linked fatty acids by treatment with mild alkali drastically reduces the biological activity of lipopolysaccharides; this indicates that fatty-acid residues are im-

portant for endotoxicity [19]. However, it seems that not only the quantity but also the nature of the ester residues is of importance, and there are some preliminary indications that the presence or absence of acylated hydroxy fatty-acid esters may determine the degree of endotoxicity of a lipopolysaccharide (E. T. Rietschel, unpublished observations).

On the basis of our present knowledge of the structure of lipid A, an atomic model of an Ra lipopolysaccharide has been built (figure 4). It can be seen that the oligosaccharide extends in one direction, while the fatty acids can easily be directed to the other. A corresponding formula of the inner-core lipid A region of salmonella lipo-

Table 1. Summarized data on fatty-acid composition of lipid A from various sources [17, 18].

Fatty acid*	Source
C12, C14, C16	<i>Salmonella</i> [2, 41-44]
3-OH-C14	<i>Escherichia coli</i> [45-51]
	<i>Proteus mirabilis</i> [52]
	<i>Aerobacter aerogenes</i> [53, 54]
	<i>Bordetella pertussis</i> [48]
C12, C14, C16	<i>Serratia marcescens</i> [55-57]
3-OH-C12	<i>Neisseria perflava</i> [58]
3-OH-C14	
C12 (C16)	<i>Pseudomonas aeruginosa</i> [59-61]
3-OH-C10	<i>Pseudomonas alcaligenes</i> [62]
(2-OH-C12)	<i>Azotobacter agilis</i> [63]
3-OH-C12†	
C12	<i>Rhodopseudomonas capsulata</i> [64]
3-OH-C10	
3-OH-C14	
C13	<i>Veillonella</i> [65, 66]
3-OH-C13	
3-OH-C15	
(C16), C15,† C17‡	<i>Myxococcus fulvus</i> [67]
3-OH-C16	<i>Polyangium</i> [67]
3-OH-C15‡	<i>Flexibacter</i> [67]
3-OH-C17‡	<i>Cytophaga</i> [67]
C16, C17, C18	<i>Brucella melitensis</i> [68]
	<i>Brucella abortus</i> [69]
C12, C16, C18, C22	<i>Anacystis nidulans</i> [70]
3-OH-C14	

* The number that follows the letter C indicates the number of carbon atoms in each fatty acid. Thus, C14 is myristic acid; 3-OH-C14 is 3-hydroxymyristic acid.

† Bold-face type indicates amide-linked fatty acids.

‡ Iso-branched fatty acids.



Figure 4. Atomic model of the partial structure of a salmonella Ra lipopolysaccharide. Shown is an acylated diglucosamine unit (lipid A unit) carrying the Ra oligosaccharide at position 3', and a second acylated diglucosamine unit linked at position 1 through a pyrophosphate bridge.

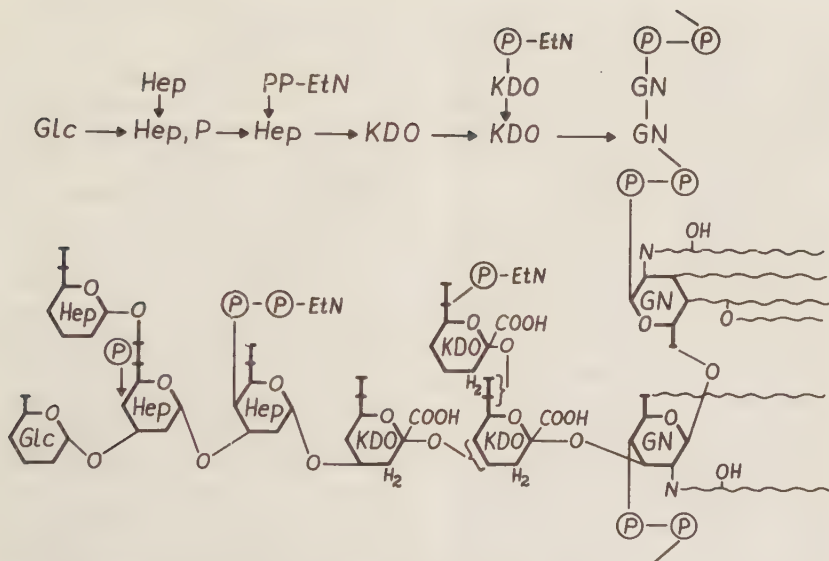


Figure 5. Structure of the inner core and lipid A of a salmonella Rc glycolipid. The presence of a third heptose unit and a phosphate group on heptose II was identified recently in mutants of higher R classes (V. Lehmann, unpublished observations). 4-Amino-arabinose, which occurs in salmonella glycolipids in an unknown linkage, is not shown [2].

polysaccharides is shown in figure 5. A series of partial degradation products of the lipid A part of salmonella glycolipids, obtained after treatment with sodium hydroxide, hydroxylamine, or hydrazine, has been identified. The changes in structure after such treatments are given in table 2. From these compounds the corresponding sugar-free lipid A fragments can be obtained by treatment with acetic acid; this results in a cleavage of the KDO linkages and an additional partial cleavage of pyrophosphate linkages.

Possible linkage of lipopolysaccharide to other cell-wall constituents. The fact that defective lipopolysaccharides (glycolipids) from R mutants can be extracted with a mixture of phenol-chloroform-petroleum ether (PCP) under very mild conditions (4 C) indicates that glycolipids are not covalently linked to the bacterial cell wall [8]. On the other hand, S-form lipopolysaccharides, which are not soluble in PCP, are often extracted with

Table 2. Series of available degradation products of lipid A derived from a glycolipid of *Salmonella*.

Treatment of glycolipid	Changes in structure
None	See formula, figures 3 and 6
0.25 N NaOH, 56 C, 5 min	Loss of (mainly) myristoxymyristic acid [17]
0.25 N NaOH, 56 C, 60 F	Partial (about 50%) loss of ester-linked fatty acids [17, 19]
Hydroxylamine	Loss of ester-linked fatty acids, and, possibly, cleavage of pyrophosphate linkages [17]
Hydrazine (2 hr)	Loss of ester-linked and partial loss of amide-linked fatty acids (figure 1C)
Hydrazine (12 hr)	Loss of all fatty acids and cleavage of pyrophosphate bridges (figure 1A)

phenol and water at 68 C [7]; in this case linkages may be cleaved. Other, mostly milder, methods of extraction often yield preparations containing lipopolysaccharide, protein, and cephalin (trichloroacetic acid [20], ethylene glycol [21]). Although it has been assumed that these mixtures represent complexes formed by hydrophobic interactions, the existence of covalent linkages between the components could not be excluded.

Recently, Wober and Alaupović [22, 23] and Tarcsay et al.¹ have studied such "complexes." From their results with *S. marcescens*, the authors concluded that the lipopolysaccharide is covalently linked to protein through lipid A, probably by a N-glycosidic linkage of reducing-terminal glucosamine residues to asparagine.

On the basis of these findings, it seems possible that lipopolysaccharide is synthesized on protein-linked lipid A. In subsequent steps the linkage to protein would be cleaved and the S or R lipopolysaccharide transported to the outer wall. In this case only a small fraction of lipopolysaccharide would be found to be linked to membrane protein. Alternatively, one could assume that lipopolysaccharide is synthesized in the membrane and is not attached to protein. After transport to the surface, only completed S lipopolysaccharide is linked to protein.

Studies analogous to those of Wober, Tarcsay, and Alaupović [22, 23]² are presently being done on R mutants.

Biosynthesis of lipid A. While the biosynthesis and genetics of the O-specific chains and the core of salmonella lipopolysaccharides have been studied intensively, not much is known about how lipid A is synthesized. Heath et al. [24] have demonstrated the transfer of KDO from cytidine monophosphate-KDO to a fragment of lipid A identified as an oligomer of N-3-hydroxymyristoyl-glucosamine. These results may suggest that addition of KDO occurs at an early stage in synthesis of lipid A, before incorporation of ester-linked fatty acids. Later, Taylor and Heath [25] studied the enzymatic incorporation of 3-hydroxy fatty acids from the corresponding acyl-carrier-protein derivative into a phospholipid of *E. coli* B. The authors discuss the possibility that this phospho-

lipid may function as an acyl donor for glucosamine residues of lipid A.

Very recently, Humphreys and Meadow [18, 26] demonstrated the incorporation of 3-hydroxylauric acid from 3-hydroxylauryl-coenzyme A into an EDTA preparation of *Pseudomonas alcaligenes* in whose lipid A component this is the main hydroxy fatty acid. On the basis of their results, obtained with the radioactive lipopolysaccharide isolated from the incubation mixture, the authors concluded that the ¹⁴C-hydroxy fatty acid was incorporated into lipid A.

We have shown recently that an enzyme preparation of a mutant of *Salmonella typhimurium* defective in glucosamine-6-phosphate deaminase [27] catalyzes the synthesis of uridine diphosphate-glucosamine from uridine triphosphate, adenosine triphosphate, and glucosamine (M. Simon, unpublished observations). We are investigating the question of whether UDP-GlcN may function as a precursor for the synthesis of the lipid A backbone.

Neither KDO- nor lipid A-defective mutants have been isolated so far. It seems that the inner regions of lipopolysaccharides have functions important for the cells and are necessary for their survival. N-acetylglucosamine-negative mutants have been described; they are defective in glucosamine-6-phosphate deaminase [27]. Recently, mutants lacking glucosamine-6-phosphate synthetase were identified [28, 29]. These mutants should be useful for studying early steps in the biosynthesis of lipid A and for investigating the question of whether a lipid A pool exists in normal, growing cells.

Biological Activity of Lipid A

Anticomplementary activity of lipopolysaccharides. It has long been known that lipopolysaccharides can interact with complement in vitro in the absence of added antibody, resulting in loss of hemolytic activity. It was also suggested that the inactivation of complement by lipopolysaccharide might be a measure of its endotoxicity, because certain biological activities that are manifested in vivo during infection by gram-negative bacteria were also shown to follow the in-vitro interaction of complement with lipopolysaccharide [32].

¹ L. Tarcsay, C. S. Wang, and P. Alaupović, manuscript in preparation.

² See footnote 1.

We have tested the anticomplementary activity of a large number of S and R lipopolysaccharides derived from *Salmonella*, *E. coli*, and *Arizona* ([33] and C. Galanos, unpublished observations). Only about one third of these proved to be highly anticomplementary (figure 6). The others exhibited only low anticomplementary activity, no matter how much lipopolysaccharide was used. In fact, after a maximum was reached with a given amount of lipopolysaccharide, addition of more resulted in fixation of less complement; (figure 6, curve B). Therefore, the difference between "active" and "inactive" lipopolysaccharides is qualitative and not quantitative. The physicochemical state of a lipopolysaccharide, the presence of divalent ions and basic amines, and the batch of guinea-pig serum used may influence the anticomplementary activity of a lipopolysaccharide. Further studies on these phenomena are under way.

We investigated the presence of natural antibodies in the guinea-pig serum used as a source of complement; these antibodies may be partly involved in the interaction of complement and lipopolysaccharide. This was done by tests of the hemolytic activity of the guinea-pig serum with ovine erythrocytes that were passively sensitized with various alkali-treated lipopolysaccharides. It

is known that treatment with alkali abolishes the anticomplementary activity of a lipopolysaccharide [33]. Titers ranging from 1:8 to 1:128 were found against 52 of 54 lipopolysaccharide preparations obtained from *Salmonella*, *Arizona*, and *E. coli*. It is evident that natural antibody activity in guinea-pig serum is at least partly involved in the interaction of complement with lipopolysaccharide (C. Galanos, unpublished observations). It was shown that free solubilized lipid A (see below) obtained by acid hydrolysis of various lipopolysaccharides (in complex form with solubilizing carriers) was highly anticomplementary, even when lipid A was obtained from lipopolysaccharides which themselves were inactive [33]. Therefore, the region in the lipopolysaccharide molecule responsible for anticomplementary activity is the lipid A.

Endotoxic activity of lipid A. We used two approaches to identify the biologically active center of lipopolysaccharides. First, we modified chemically the KDO residues in an Re glycolipid to determine whether or not the oligo(poly)saccharide portion of lipopolysaccharides plays a specific role in endotoxicity. Secondly, free lipid A was tested directly for endotoxic activity.

Table 3 shows the results obtained with chem-

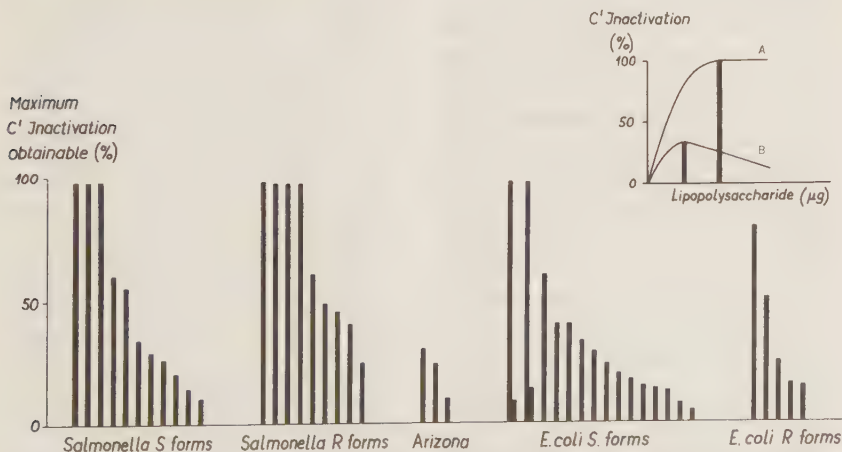


Figure 6. Anticomplementary activity of various lipopolysaccharides with a given guinea-pig serum. Each column denotes maximal activity obtainable with each preparation that cannot be surpassed by higher concentrations of lipopolysaccharide [33]. The two curves are meant to illustrate this.

Table 3. Biological properties of unaltered and modified Re glycolipid of *Salmonella minnesota*.

Glycolipid	HAI ($\mu\text{g/ml}$)*	50% inactivation of complement (μg)	Mouse lethality $\text{LD}_{50}^{\dagger}$ (μg)	Chick-embryo lethality LD_{50} (μg) ‡	Pyrogenicity LD-3§ ($\mu\text{g/kg}$)
Unaltered	8	4	15	0.006	0.0035
N-dinitrophenylated	2	5	20	0.001	ND
O- and N-succinylated	>250 (16)**	22	12	ND	0.002
Carboxymethylated	>250 (8)**	5	80	ND	ND

* Minimal amount of inhibitor that prevented hemagglutination.

 † Mouse lethality tests were performed at 37 C. ‡ Tests performed in Dr. A. Nowotny's laboratory.

§ Test performed in laboratory of Drs. D. W. Watson and Y. B. Kim. LD-3 = minimal pyrogenic dose after 3 hr.

|| ND = not done.

** Preparation tested after treatment with alkali.

ically modified glycolipid [30]. Amino groups (present in the form of KDO-linked phosphoryl-ethanolamine residues) were dinitrophenylated, hydroxyl groups (and amino groups) of KDO (and ethanolamine) were succinylated, and the carboxyl groups of KDO were converted into their methyl esters. It was found that these modifications did not significantly change the activity of the glycolipid when tested for lethality to mice or chick embryos, pyrogenicity, or inactivation of complement. However, the serologic activity of the parent glycolipid was completely lost after succinylation and carboxymethylation. That this was due to the substituents introduced into the molecule could be shown by treatment with alkali, which simultaneously removed the substituents and restored full serologic activity [30]. These results showed that chemical modification of the oligosaccharide portion, even to a degree where the original serologic specificity was completely lost, did not result in a significant alteration of the endotoxic activity of the glycolipid. It is concluded that the polysaccharide part of lipopolysaccharides is not specifically involved in endotoxicity.

A new approach was used to demonstrate directly the role of lipid A as the endotoxic principle. This made it possible to obtain free lipid A in a stable, soluble form [3, 31, 33]. Lipid A as obtained from lipopolysaccharides (R form) after hydrolysis with mild acid is insoluble in water and, therefore, biologically inactive. It was found that lipid A can be solubilized by complexing with certain carriers that are water-soluble and nontoxic by themselves. Complexes of lipid

A with alkali-treated lipopolysaccharides, alkali-treated lipid A, or serum albumin were prepared [33]. For demonstration of their biological activity, inactivation of complement was used as a convenient in-vitro test. The complexes mentioned above were found to be highly active in this test. Subsequent investigations with complexes of lipid A and serum albumin revealed endotoxic activity in a number of in-vivo and in-vitro tests [33, 34]. Table 4 shows that the following assays were performed: mouse lethality, pyrogenicity [34], bone-marrow necrosis,³ Limulus gelation [35], and complement inactivation [34]. Although the lipid A complexes were found to be in general somewhat less active than the corresponding parent lipopolysaccharides, the results strongly indicate that lipid A represents the biologically active center in endotoxic lipopolysaccharides and that the polysaccharide component acts as a solubilizing carrier that can be replaced by other, noncovalently bound carriers like serum albumin.

Recently, the toxicity of lipopolysaccharide from an Rb mutant of *Salmonella minnesota* and the serum-albumin complex of its lipid A was measured in adrenalectomized mice; LD_{50} values of 0.075 μg and 0.02 μg , respectively, were obtained (C. Galanos and M. Parant, unpublished observations).

Preparation and properties of antiserum to lipid A. Immunologic studies on the lipopolysaccharides (O antigens) of gram-negative bacteria have

³ M. Yoshida, C. Galanos, E. T. Rietschel, and O. Lüderitz, manuscript in preparation.

Table 4. Biological properties of lipid A-bovine serum albumin complexes as compared with the parent lipopolysaccharides (glycolipids).

Preparation		Mouse lethality LD ₅₀ (μ g)	Pyrogenicity LD-3* (μ g/kg)	Bone-marrow reaction ED ₅₀ [†] (μ g)	Complement inactivation [‡] (%)	Limulus-lysate gelation [35] (μ g/ml)
<i>Salmonella minnesota</i> R60	LPS§	300	...	...	30	
	LA/BSA	1,700	0.003	18.8	86	
<i>S. minnesota</i> R345	LPS	300	...	6.2	5	
	LA/BSA	1,700	0.013	7.2	85	10 ⁻⁸
<i>S. minnesota</i> R5	LPS	650	...	...	70	10 ⁻¹²
	LA/BSA	4,000	0.007	10.9	85	
<i>S. minnesota</i> R595	LPS	1,750	0.004	3.9	80	10 ⁻¹²
	LA/BSA	4,000	0.007	11.0	86	
<i>Escherichia coli</i> EH100	LPS	...	...	...	15	
	LA/BSA	1,700	...	...	80	
BSA control		>10,000	>100	>100	0	>10 ⁻³

* LD-3 = minimal pyrogenic dose after 3 hr; data from [34].

† ED₅₀ = 50% effective dose.‡ With 10 μ g of preparation [33].

§ LPS = lipopolysaccharide; LA = lipid A; BSA = bovine serum albumin.

|| Larger amounts of these preparations do not lead to inactivation of more complement; in fact, smaller amounts are inactivated.

revealed that distinct structures in the O-specific polysaccharide chains function as the serologic determinants against which the O antibodies are directed. In incomplete lipopolysaccharides of R mutants, oligosaccharides of the basal core represent the determinant regions [2]. In addition to these specific O and R antigens, a number of antigens present on the enterobacterial cell have been described that, under certain conditions, exhibit common specificities, thus causing cross-reactions between species [36]. The best known example is the common antigen of Kunin [37], which is present on the surface of all Enterobacteriaceae. However, this antigen is detectable only with antisera obtained with a limited number of species that carry the common antigen in an immunogenic form. In most strains, its immunogenicity is not expressed due to the presence of lipopolysaccharide [38].

A similar situation seems to exist with regard to lipid A, which is a common component of all lipopolysaccharides and is structurally identical or similar in all Enterobacteriaceae [3]. The formation of antibodies against this common structure of lipopolysaccharides might be expected, but such antibodies were not described previously. In fact, results indicate that the formation of antibodies to

lipid A is suppressed to a large extent when immunization is performed with lipopolysaccharides or bacterial cells, in which lipid A carries long polysaccharide chains (S forms) or oligosaccharide units (R mutants). Only recently it was shown that lipid A, when presented to the animal (rabbit) in a suitable form, can act as an immunogen. It was found that immunization with intact *S. minnesota* R595 bacteria, which have the glycolipid KDO-lipid A on their cell surface, resulted in low levels of antibodies to lipid A. Improved titers were obtained by immunization with the same bacteria from which KDO had been cleaved by hydrolysis with mild acid (figure 7). Such hydrolyzed bacteria contain free lipid A in their outer cell membranes. These hydrolyzed bacteria, when coated with additional lipid A that was isolated from a lipopolysaccharide by hydrolysis with mild acid and used for immunization, elicited the highest titers of antibody to lipid A so far obtained (figure 7). Formation of antibody shows a time course resembling that of antibody formation against the polysaccharide structure of lipopolysaccharides [39]. The results show that lipid A, when sufficiently exposed on the bacterial cell, stimulates the formation of specific antibodies in the animal.

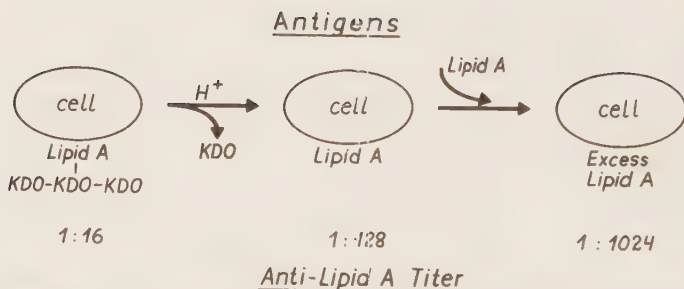


Figure 7. Preparation of immunogenic lipid A. Modification of Re mutant cells and titers of antibody to lipid A obtained with unaltered cells, hydrolyzed cells, and hydrolyzed cells complexed with lipid A [36].

For determination of anti-lipid A activity in such antisera, the passive-hemolysis test with human red blood cells sensitized to lipid A was used. The sensitivity of the test can be increased significantly by use of ovine erythrocytes (and the antisera absorbed with these cells). Another specific assay for determining anti-lipid A activity involved liposomal model-membranes [39]. Lipid A-sensitized erythrocytes were also used for the absorption of antibodies to lipid A from immune sera and their reisolement. The specificity of the immune sera for lipid A could thus be demonstrated.

Preparations of lipid A obtained from one *E. coli* and a number of salmonella lipopolysaccharides exhibit strong serologic cross-reactions with different antisera to lipid A. All lipopolysaccharides tested cross-react with the antiserum to lipid A, some to a high degree. Similarly, many antibacterial antisera and "normal sera" exhibit activity against lipid A. This means that although lipid A is not always sufficiently exposed on the surface of the bacteria for induction of antibodies, antibodies to lipid A are capable of reacting with lipopolysaccharides, even with those of S forms. It is well known that antibodies directed against surface structures of the bacterial cell play an important role in defense mechanisms against infection [40]. The finding that antibodies to lipid A are capable of reacting with lipopolysaccharides indicated that they might function as opsonins and promote phagocytosis of bacteria. One system has been studied so far. *E. coli* 0111 were treated with antiserum to lipid A, and their fate in the peritoneum of the mouse was followed. It was found that antibodies to lipid A were effective at high

dilutions in sensitizing these bacteria for subsequent phagocytosis and killing [36]. Recent studies have shown that antibodies to lipid A also enhance the blood clearance of heat-killed *Salmonella enteritidis* (C. Galanos and M. Parant, unpublished observations).

Thus, antibodies to lipid A are capable of reacting with lipopolysaccharides on the cell surface of S-form bacteria. How far this is true for Enterobacteriaceae in general is a very important question. Since all enterobacterial cell walls contain lipid A, highly specific lipid A antiserum may be found to be reactive universally with a great number of otherwise serologically distinct species. Studies are under way to find out whether anti-lipid A antibodies can afford protection to enterobacterial infections.

Possible interactions of antibodies to lipid A with the endotoxic properties of lipopolysaccharides or lipid A are another important subject for investigation.

We have preliminary indications that amide-linked 3-hydroxymyristic acid functions as an immunodominant group in lipid A.

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Interaction of Lipopolysaccharides and Lipid A with Complement

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A number of lipopolysaccharides derived from *Salmonella* and *Escherichia coli* S and R mutant strains were tested for toxicity and anticomplementary activity in the absence of added antiserum. Although all preparations were toxic, only a few exhibited high anticomplementary activity, while others proved to be of low or negligible activity. It was found that isolated lipid A from both active and inactive lipopolysaccharides was strongly anticomplementary as well as toxic, when made water-soluble with the aid of suitable carriers. Treatment of R form lipopolysaccharide with Mg^{2+} or Ca^{2+} led to complete precipitation of the lipopolysaccharide with consequent loss of toxicity and anticomplementary activity. This treatment had practically no effect on the anticomplementary activity and toxicity of S form lipopolysaccharides. When lipopolysaccharide, after reaction with complement, was reisolated and purified, the resulting preparation was found to be non-toxic and of negligible anticomplementary activity. No detectable alterations in either the sugar or the fatty acid composition could be detected. The only significant change was the loss of solubility in water. Treatment of the reisolated lipopolysaccharide with EDTA completely restored solubility in water, toxicity, and anti-complementary activity.

It has been known for a long time [1] that the cell wall lipopolysaccharides of gram-negative bacteria exhibit anticomplementary activity. Recently, Mergenhausen *et al.* [2,3] have shown that complement-endotoxin interaction leads to loss of terminal complement activity with simultaneous appearance of lesions on the lipopolysaccharide molecule as judged by electron microscopy. In experiments *in vitro*, it could also be demonstrated that complement-endotoxin interaction leads to certain biological activities such as the generation of anaphylatoxin, chemotactic factors and the activation of the clotting system [3,4]. Since these phenomena are also observed after injection of endotoxin in animals, it has been suggested [3] that some of the endotoxic activities of lipopolysaccharides are mediated through the activation of the complement system. The above studies were primarily concerned with the role of complement in complement-endotoxin interactions and the biological significance of this phenomenon.

In this paper the emphasis is directed mainly towards the role and fate of the lipopolysaccharide in reactions involving complement, and in particular to the region of the molecule responsible for anticomplementary activity. Previous studies have shown that for anticomplementary activity [2] and for toxicity the O-specific chains of lipopolysaccharides as well as part of their core polysaccharide are not necessary; i. e. R form lipopolysaccharides con-

taining lipid A and 2-keto-3-deoxyoctonate (KDO) as the main constituents can be as active as the parent S form lipopolysaccharides. The role of lipid A in these reactions became thus more apparent.

In the present study further evidence will be supplied that lipid A is the region in lipopolysaccharides which is responsible for both lethal toxicity in mice and for anticomplementary activity. Lipid A is present in all lipopolysaccharides. However, screening of a number of toxic water-soluble lipopolysaccharides, showed that some were anticomplementary and others were not. The reason for this is still unknown. The influence of solubility on the anticomplementary activity of lipopolysaccharides was studied. It was found that when R form lipopolysaccharides possessing both anticomplementary activity and toxicity were precipitated by divalent cations, both of these activities were lost, but could be restored by solubilization after EDTA treatment. Finally, it was shown that lipopolysaccharide-complement interaction does not lead to any detectable changes in either the lipid A or the polysaccharide part of the lipopolysaccharide molecule.

MATERIALS AND METHODS

Bacteria

The strains *S. minnesota* S (S form), R5, R595 and *S. godesberg* R15 (R forms) were obtained from J. Schlosshardt (Zentrallaboratorium f. bakt. Darm-infektionen, Potsdam, DDR) [5], *S. minnesota* R345

Unusual Abbreviation. KDO, 2-keto-3-deoxyoctonate.

and R60 (R forms) from F. Kauffmann (Statens Seruminstitut, Copenhagen, Denmark) [5], the other *Salmonella* S form strains from R. Rohde (Nat. Salmonella Zentrale, Hamburg, BRD), *S. typhimurium* TV 161 (R form) from B. A. D. Stocker (Stanford University, Stanford, U.S.A.) [6], and the *Escherichia coli* strains, *E. coli* 0100 (S form) and *E. coli* EH100 (R form) [7] from P. H. Mäkelä (Valtion Serumlaitos, Helsinki, Finland). The bacteria were cultivated as described by Schlecht *et al.* [8].

Lipopolysaccharide and Lipid A

The lipopolysaccharides of *Salmonella* S forms were obtained by extraction of the bacteria with phenol/water [9], those of the *Salmonella* R mutants by extraction with phenol—chloroform—petroleum ether [10].

Lipid A was prepared from the respective lipopolysaccharides by acetic acid hydrolysis (1% acetic acid, 100°C, 4 h). The precipitate formed was washed three times with distilled water and once with acetone and dried *in vacuo*. The preparations were analyzed for KDO, the absence of which indicated that the lipid A preparations were free of carbohydrates.

Alkali-treated lipopolysaccharide was prepared by heating lipopolysaccharide in 0.25 N NaOH at 56°C for 1 h [11]. The solution was acidified with acetic acid, centrifuged and the supernatant dialyzed against distilled water. After re-centrifugation the clear supernatant was freeze-dried.

Alkali-treated lipid A was obtained by heating lipid A in 0.25 N NaOH at 100°C for 20 min. The mixture was neutralized with acetic acid and dialyzed extensively against distilled water. To the inner dialysate EDTA was added to a concentration of 0.1 M (pH 8) and the mixture was further dialyzed against distilled water. Any insoluble material was removed by centrifugation and the clear solution was freeze-dried.

Analytical Methods

KDO was determined by the thiobarbituric acid method [12], glucosamine in acid hydrolysates (4 N HCl, 20 h, 100°C) by the Morgan-Elson method as described by Strominger *et al.* [13], and organic phosphate according to Lowry *et al.* [14]. The amount of Ca^{2+} plus Mg^{2+} in serum was determined according to the method of Sobel and Hanok [15].

The fatty acid content was estimated by weighing chloroform extracts of acidified alkali hydrolysates of the preparations (4 N NaOH, 5 h, 100°C). The free fatty acids were methylated with a solution of BF_3 in methanol (14% w/v; Serva, Heidelberg) [16] and applied to gas liquid chromatography in a Perkin-Elmer fractometer 20 with a flame ionization detector

on a column (1.5 × 1/8 in) containing 2.5% castorwax on chromosorb G (80/100 mesh).

The Complement Fixation Test

Guinea pig serum was obtained from the Hygiene Institute, University of Freiburg and stored at -80°C. Complement fixation was determined as follows: an amount of 100 to 150 μl undiluted serum (depending on the activity of the batch) was mixed with graded amounts of lipopolysaccharide or lipid A which were dissolved or suspended in a maximum volume of 20 μl of distilled water. The mixtures, after incubation for 1 h at 37°C, were diluted to 300 μl with veronal buffer [26]. From each solution 7 μl were added to 1 ml of the same buffer plus 0.5 ml of a suspension of sheep erythrocytes sensitized with amboceptor. After incubation at 37°C for 1 h, the samples were centrifuged and the absorbance of the supernatants were measured at 546 nm. Under these conditions the complement control (no lipopolysaccharide added) gave an absorbance of 0.8, while the erythrocyte control (0.5 ml of the erythrocyte suspension lysed with either distilled water or with excess complement in a total volume of 1.5 ml) gave an absorbance of 1.3. Anticomplementary activity is expressed as percentage inhibition of hemolysis obtained with the respective amounts (μg) of lipopolysaccharide added to the undiluted serum (100 to 150 μl).

For preparative studies, fresh pig serum was used.

Antisera

Antibacterial rabbit antisera were obtained as described previously [17].

Toxicity Test

NMRI female white mice weighing 16–20 g were used. The test substance was injected intraperitoneally in 0.5 ml distilled water, using groups of 10 mice. Deaths were recorded up to 72 h later. The LD_{50} was calculated according to Reed and Muench [24].

RESULTS

ANTICOMPLEMENTARY ACTIVITY OF LIPOPOLYSACCHARIDES

A Survey of S and R Form Lipopolysaccharides

The anticomplementary activity of *S. minnesota* S and *S. minnesota* R595 lipopolysaccharides is shown in Fig. 1, where the percentage inhibition of hemolysis is plotted against the amounts of lipopolysaccharides used. In the case of the smooth form, 3.2 μg was sufficient to produce 50% inhibition of the hemolytic complement activity. In the case of the R form the amount needed was 6.5 μg . These results agree in

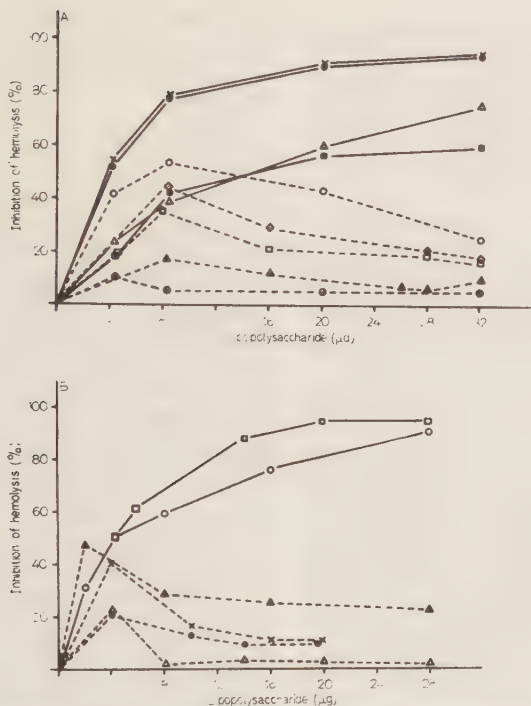


Fig. 1. Anticomplementary activity of (A) S and (B) R form lipopolysaccharides. Increasing amounts of the different lipopolysaccharides (abscissa) were incubated with 100 µl of guinea pig serum and the hemolysis tests performed as described in Methods. The S form lipopolysaccharides (A) were derived from *S. minnesota* (×), *S. raistr* (●), *S. typhimurium* (Δ), *S. abortus equi* (■), *S. typhi* (○), *E. coli* 0100:K⁺ (◊), *E. coli* 0100:K⁺ (□), *S. milwaukee* (▲), *S. godesberg* (◌). The R form lipopolysaccharides (B) were derived from *S. minnesota* R5 (□), *S. minnesota* R595 (○), *S. minnesota* R60 (▲), *S. typhimurium* TV161 (×), *E. coli* EH100 (●), *S. minnesota* R345 (Δ).

principle with those reported by Mergenhagen *et al.* [2] who used the same lipopolysaccharide preparations. However, quite different results were obtained when other S and R form lipopolysaccharide were tested for their anticomplementary activity. Out of the 9 S form lipopolysaccharides derived from different bacterial strains, only 2 proved to be highly active, while 5 exhibited medium activity and 2 were almost inactive (Fig. 1A). Similarly, the various R lipopolysaccharides tested showed a spectrum of activity, 2 being most active, 2 having low activity and 2 being inactive (Fig. 1B).

As stated in Methods, the cultivation of the bacterial strains and the extraction of lipopolysaccharides had been performed under standardized conditions.

Nevertheless, accidental differences in physical properties of the lipopolysaccharide preparations may cause differences in their anticomplementary activity. It was found that the activity of a given lipopolysaccharide may vary when different batches of guinea pig serum are compared. Thus, the lipopolysaccharide of *S. abortus equi* was highly active in a second serum batch, indicating that possibly the presence of natural specific antibodies may play a role. Support for this assumption was obtained, when lipopolysaccharides, which were found to be inactive in the above test, were assayed similarly but in the presence of specific antiserum. Mixtures of lipopolysaccharides (22 µg each of *S. minnesota* R345 and R60) and graded quantities of specific antisera (1 to

30 μ l) were added to 100 μ l of guinea pig serum. The hemolysis test was then carried out in the usual way. 50% inhibition of hemolysis was obtained with 20 μ l (R345) and 4 μ l (R60) of the respective antiserum. Similarly, inactive S form lipopolysaccharides, when tested in the presence of antiserum, showed high anticomplementary activity. Addition of normal rabbit serum did not lead to complement fixation.

Effect of Mg^{2+} and Ca^{2+} on the Anticomplementary Activity of S and R Form Lipopolysaccharides

It is known that Mg^{2+} and Ca^{2+} precipitate R form lipopolysaccharides from their aqueous solutions [18]. This precipitation is very slow at 0°C and almost instantaneous at 45°C. 10 μ mole of $MgCl_2$ or $CaCl_2$ completely precipitate 1 mg *S. minnesota* R595 lipopolysaccharide from 0.5 ml distilled water. This concentration of ions, however, has no effect on the solubility of the corresponding S form lipopolysaccharide.

The effect of Mg^{2+} and Ca^{2+} ions on the anticomplementary activity of both lipopolysaccharides was tested as follows. To the respective solutions of both *S. minnesota* S and R form lipopolysaccharides (1 mg/0.5 ml distilled water) 10 μ mole $MgCl_2$ were added at 0°C. In the case of the S form increasing amounts of lipopolysaccharide were transferred into tubes and incubated at 45°C for 5 min. With the R form, samples containing increasing amounts of lipopolysaccharide were transferred into tubes in duplicate. One series was incubated at room temperature for 5 min and the second series at 45°C for the same time. All samples (S and R) were then incubated with 100 μ l undiluted complement at 37°C for 1 h and the hemolysis test carried out as described before. The results are shown in Fig. 2. The anticomplementary activity of the S form lipopolysaccharide remains practically unaltered in the presence of Mg^{2+} .

In contrast to the smooth form, a dramatic loss of anticomplementary activity can be observed when

R form lipopolysaccharide is treated with Mg^{2+} . This was most pronounced after incubation at 45°C, where precipitation was almost complete. In a separate experiment it was shown that Ca^{2+} has the same effect as Mg^{2+} .

The serum used in these studies as a source of complement contained 0.38 μ mole calcium and magnesium per 100 μ l. As shown in the previous section, this amount of cations, when added to 25 μ g of R lipopolysaccharide in the absence of serum, would cause almost quantitative loss of anticomplementary activity. In order to test the interaction of R lipopolysaccharides with Mg^{2+} dissolved in the serum, the following experiments were carried out. 18 μ g R form and 7 μ g S form lipopolysaccharide (these quantities give in both cases approximately 95% inhibition) were added to 100 μ l quantities of the undiluted serum, to which increasing amounts of $MgCl_2$ had been added. For comparison, the same quantities of $MgCl_2$ and lipopolysaccharide were mixed, incubated at 37°C for 5 min and added to 100 μ l of undiluted serum. All samples were then incubated at 37°C for 1 h, diluted to 300 μ l with buffer and tested in the usual way.

The results are shown in Fig. 3. As expected a rapid loss of anticomplementary activity could be seen when R595 was preincubated with Mg^{2+} . An amount of 0.1 μ mole of Mg^{2+} per 1 μ g of this lipopolysaccharide suppressed the anticomplementary activity by more than 80%. This loss of activity was much less pronounced when Mg^{2+} was added to the serum. Nevertheless, 0.1 μ mole of Mg^{2+} resulted in 50% loss of anticomplementary activity. Since this amount of ions is comparable to that already present in serum, it may be assumed that the activity of R form lipopolysaccharides is higher than measurements show, since they may be partly precipitated by the ions naturally present in serum, hence not participating in the interaction with complement.

In the case of S form lipopolysaccharide, with amounts of Mg^{2+} up to 0.1 μ mole per 1 μ g lipopolysaccharide, no detectable loss of activity occurred

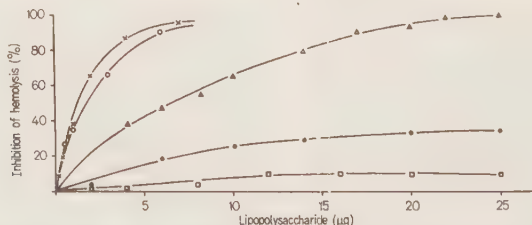


Fig. 2. The anticomplementary activity of Mg^{2+} -treated lipopolysaccharides. The lipopolysaccharides were pretreated with $MgCl_2$ and graded amounts of the mixtures were tested for complement inactivation. *S. minnesota* S form lipopolysaccharide control (x) and incubated with Mg^{2+} for 5 min at 45°C (○), *S. minnesota* R595 lipopolysaccharide control (Δ) and incubated with Mg^{2+} for 5 min at 22°C (●) and 45°C (□).

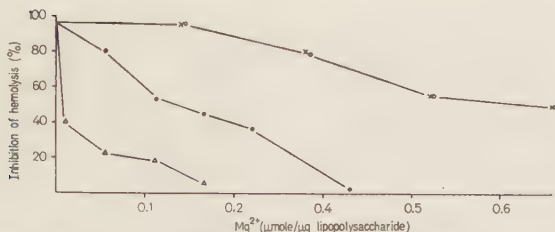


Fig. 3. The effect of Mg^{2+} added to guinea pig serum on the anticomplementary activity of lipopolysaccharides. Constant amounts of the lipopolysaccharides (causing about 95% inhibition of hemolysis) were added to 100 μ l of serum containing graded quantities of $MgCl_2$. The hemolysis test was then carried out in the usual way: *S. minnesota* S form (O) and R595 (●). For comparison, the lipopolysaccharides were pretreated with the same amounts of $MgCl_2$ and the mixtures added to the serum: *S. minnesota* S form (x) and R595 (Δ).

(see Fig. 3). Some loss of activity could be seen with higher amounts of Mg^{2+} .

The results show that R lipopolysaccharides, when precipitated with divalent cations lose their anticomplementary activity. S form lipopolysaccharides are neither precipitated nor inactivated by these cations.

ANTICOMPLEMENTARY ACTIVITY OF SOLUBILIZED LIPID A

Soluble Lipid A Preparations

It has already been shown [2] that for complement inactivation by endotoxin the O-specific chains as well as part of the core are not necessary, and that the lipopolysaccharide of *S. minnesota* R595, containing mainly KDO and lipid A, exhibits high anticomplementary activity. Free lipid A, however, obtained by acid hydrolysis is insoluble in water, and does not show any anticomplementary activity.

Lipid A can be solubilized by several disaggregating agents, such as triethylamine [19] or pyridine [20].

In the former case, addition of 5 μ l triethylamine to 2 mg lipid A in 1 ml water renders lipid A soluble. In the latter case, solubilization is achieved by dissolving lipid A in pyridine followed by dilution with water. The pyridine is then expelled under vacuum in a rotary evaporator, with water being added to replace the pyridine lost. In this way, pyridine is completely replaced by water, whereby an almost clear solution of lipid A is obtained. However, lipid A thus solubilized does not possess anticomplementary activity. Therefore, other methods of solubilization were studied, which consisted of complexing lipid A with a soluble carrier. Three different carriers have been used: alkali-treated lipopolysaccharide, alkali-treated lipid A and bovine serum albumin. All three substances were water soluble and exhibited no or

low (alkali-treated lipopolysaccharide) anticomplementary activity.

The complexing procedure was as follows: lipid A was dissolved in water by addition of triethylamine (5 μ l triethylamine/ml containing 2 mg lipid A). This solution was mixed with a solution of the respective carrier, and the mixture dried in a rotary evaporator under reduced pressure, whereby the triethylamine was completely removed. The respective complexes were redissolved in water and once more taken down to dryness. They were finally dissolved in water at 50° C and, where necessary subjected to short ultravibration.

The solubility of the complexes was dependent on the concentration of the soluble carriers present.

Lipid A/Alkali-Treated Lipopolysaccharide Complex

To find the optimum conditions for solubilization, lipid A (derived from *S. illinois* S form) and alkali-treated lipopolysaccharide (derived from *S. minnesota* S form) were complexed in different ratios. Mixtures of lipid A and alkali-treated lipopolysaccharide in the ratio of 1:4 and 1:2, respectively gave almost clear solutions. As the proportion of lipid A was increased the resulting solutions became correspondingly more opalescent, and when the amount of lipid A in the complex was higher than that of treated lipopolysaccharide the aggregate was no longer completely soluble. The anticomplementary activity of these complexes was then tested. The results are shown in Fig. 4. The most soluble complexes show the highest activity. As the solubility decreases there is a corresponding decrease in anticomplementary activity. The carrier alone (treated lipopolysaccharide) shows a very low activity. A number of other lipid A/treated lipopolysaccharide complexes in a 1:1 ratio was then tested. In each case, both parts of the com-

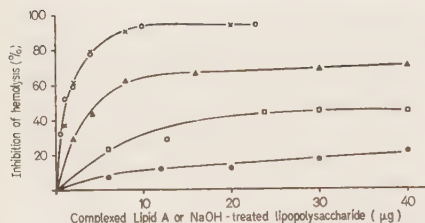


Fig. 4. Anticomplementary activity of complexes containing lipid A and alkali-treated lipopolysaccharide in different ratios. Lipid A from *S. illinois* S form and treated lipopolysaccharide from *S. minnesota* S form were complexed in ratios of 1:4 (○), 1:2 (×), 1:1 (Δ), and 1:0.3 (□); treated lipopolysaccharide control (●). The hemolysis test was carried out as usual.

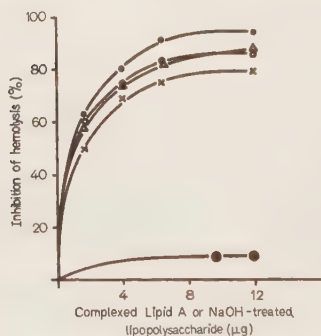


Fig. 5. Anticomplementary activity of complexes of lipid A and alkali-treated lipopolysaccharide. In each case, the two components were prepared from the same lipopolysaccharide. *S. milwaukee* S form (●), *S. minnesota* R345 (○), *S. typhimurium* TV161 (Δ), and *S. typhi* (×). As controls, the treated lipopolysaccharide preparations were tested in the absence of lipid A and the mean values are given (⊙).

plex were derived from the same lipopolysaccharide. Thus, complexing was carried out with lipid A and treated lipopolysaccharide prepared from each of the following lipopolysaccharides: *S. milwaukee* S, *S. typhi* S, *S. godesberg* S, *S. minnesota* R345, *S. typhimurium* TV161. As seen from Fig. 5, all these complexes exhibited high anticomplementary activity, even when the lipid A used was derived from a lipopolysaccharide which itself was not active.

Lipid A/Alkali-Treated Lipid A Complex

Lipid A preparations, derived from *Salmonella* R mutants (*S. minnesota* R595 and R60, *S. godesberg* R15) were complexed with the alkali-treated lipid A

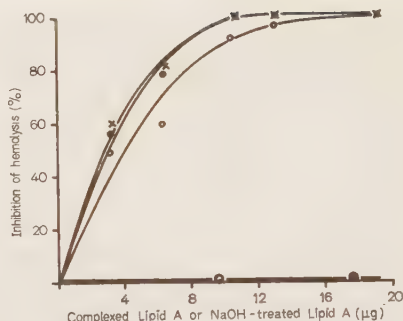


Fig. 6. Anticomplementary activity of lipid A/alkali-treated lipid A complexes. The components were present in a ratio 1:0.5 (lipid A/treated lipid A). Treated lipid A was obtained from *S. minnesota* R595 (●). The lipid A preparations used in the complexes were obtained from *S. minnesota* R595 (●), *S. minnesota* R60 (×), and *S. godesberg* R15 (○).

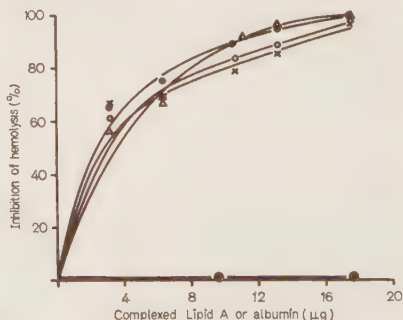


Fig. 7. Anticomplementary activity of lipid A/bovine serum albumin complexes. The components were present in a ratio 1:0.5 (lipid A/albumin). The lipid A preparations were obtained from *S. minnesota* R60 (●), *S. minnesota* R595 (Δ), *E. coli* EH100 (○), and *S. godesberg* R15 (×). Albumin was used as a control (⊙).

(treated lipid A) preparation obtained from *S. minnesota* R595. It was found that treated lipid A was a better solubilizing agent than treated lipopolysaccharide, and a ratio of lipid A: treated lipid A of 1:0.5 was adequate for the solubilization of lipid A. Fig. 6 shows the anticomplementary activity of the three complexes. All three complexes show very high activity.

It should be mentioned that the alkali-treated lipid A preparation exhibited hemolytic activity when incubated with erythrocytes in the absence of complement. This lytic activity was first observed when

Table 1. Analysis of *S. minnesota* R595 lipopolysaccharide after treatment with serum (complement), reisolation and solubilization with EDTA, in comparison with original lipopolysaccharide
n.d. = not determined

Lipopoly- saccharide <i>S. minnesota</i> R595	GlcN	P	KDO	Fatty acids					
				Total	Individual fatty acids*				
					Lauric	Myristic	Palmitic	β -Hydroxy- myristic	<i>d</i> -Tetra- decenoic ^b
	%	%	%	%	mole %	mole %	mole %	mole %	mole %
Original	13.4	3.12	17.4	53.0	17.9	18.3	19.2	30.7	13.9
Reisolated	13.3	3.30	n.d.	53.3	18.6	18.6	17.1	30.8	14.8
EDTA solubilized	13.3	2.81	17.1	56.6	n.d.	n.d.	n.d.	n.d.	n.d.

^a Determined by gas liquid chromatography.

^b This unsaturated acid arises from a O-substituted β -hydroxymyristic acid by a β -elimination reaction under the alkaline hydrolysis conditions [27].

treated lipid A was used in order to sensitize erythrocytes. However, this activity is seen only in concentrations much higher than those used in the above tests.

Lipid A/Bovine Serum Albumin Complex

Bovine serum albumin was by far the best solubilizing carrier so far tested. Soluble complexes were obtained even when the amount of lipid A in the complex was 10 times higher than that of albumin. The proportion of albumin in a lipid A complex was not found to affect the anticomplementary activity of the latter. For this reason lipid A/albumin complexes in the ratio of 1:0.5 have been used in the following experiments, where four different lipid A preparations were tested. These were derived from *S. minnesota* R595, R60 and *S. godesberg* R15 and from *E. coli* EH100. As shown in Fig. 7 the anticomplementary activity of all four complexes with albumin was very high.

REISOLATION OF R FORM LIPOPOLYSACCHARIDE AFTER INTERACTION WITH COMPLEMENT

Purification and Chemical Analysis of Reisolated Lipopolysaccharide

When complement reacts with endotoxin, the former loses its hemolytic activity. In addition it has been shown by electron microscopy that lesions ("pits") appear on the complement-treated lipopolysaccharide [2,3]. It was not clear, however, whether the above findings could be interpreted to mean that the lipopolysaccharide molecule had undergone irreversible chemical changes. To obtain information on this matter complement-treated lipopolysaccharide was reisolated from serum. The purified material was then used for chemical and biological investigations.

340 mg of *S. minnesota* R595 lipopolysaccharide were added in portions over a period of 1 h to 400 ml of pig serum at 37° C, and the mixture incubated further for 30 min. This resulted in the inactivation

of 80% of the complement activity originally present in the serum. The mixture was then centrifuged at 13000 $\times$ g for 30 min.

The sediment which contained the lipopolysaccharide was washed once with distilled water and dried *in vacuo* (yield 490 mg). The crude material was extracted by the phenol-chloroform-petroleum ether method [10], whereby the lipopolysaccharide was obtained in a pure form (280 mg, overall yield about 80%).

Chemical analysis was performed comparatively on the original and complement-treated lipopolysaccharides (Table 1). The KDO content was found to be identical in both preparations. Also no significant qualitative or quantitative differences in the fatty acid distribution could be detected. Furthermore, the glucosamine and phosphate content was the same in both preparations. From these findings it is concluded that interaction of complement and lipopolysaccharide leads to no detectable chemical alterations in either the sugar or the lipid A part of the molecule.

Anticomplementary Activity of the Reisolated Lipopolysaccharide

The product isolated after complement interaction was completely insoluble in water and, as expected, the anticomplementary activity of this material was very low (Fig. 8). The preparation was then treated with EDTA (0.2 M, pH 7.5), dialyzed extensively against distilled water and lyophilized. As a result of this treatment the material became completely water-soluble, and when its anticomplementary activity was now tested, it was found to be fully restored (Fig. 8). From this experiment it is concluded that the anticomplementary activity of endotoxin is not altered by complement interaction, and that the loss observed with the material isolated from serum before EDTA treatment was due to its insolubility in water caused by absorption of divalent cations from the serum.

In the above experiment, pig serum was used as a source of complement. Unlike guinea pig serum this serum is readily available in large quantities and is rich in terminal complement components, which are chiefly involved in the interaction with endotoxin. However, similar results were obtained in small scale experiments using guinea pig serum. Under identical conditions 67% of lipopolysaccharide was recovered after reaction. Although the amount of lipopolysaccharide used did not permit toxicity tests, chemical analysis and complement fixation tests on the reisolated insoluble material and on the solubilized material after EDTA treatment gave results identical to those obtained with pig serum.

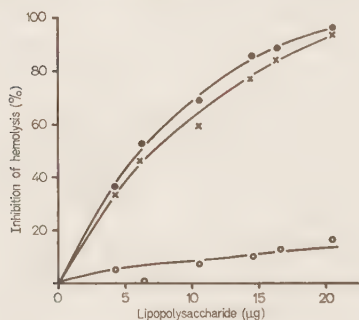


Fig. 8. Anticomplementary activity of *S. minnesota* R595 lipopolysaccharide after treatment with serum (complement). Activity of the reisolated insoluble (○), EDTA-solubilized (×) preparation, and of the original R595 lipopolysaccharide (●).

Mouse Lethality Tests

The toxicity of the material reisolated from serum was tested in mice by comparison with analogous preparations. The results are summarized in Table 2.

While the original soluble *S. minnesota* R595 lipopolysaccharide was found to have a LD_{50} of about 1750 µg, the insoluble material reisolated from serum proved to be non-lethal with amounts of up to 4000 µg. After treatment with EDTA, it became soluble and its toxicity was restored. The toxicity of Mg^{2+} and Ca^{2+} precipitated lipopolysaccharide was then tested. Excess of Mg^{2+} and Ca^{2+} was added to a solution of the R595 lipopolysaccharide and warmed to 50°C for 3 min. The resulting precipitate was centrifuged and washed twice with distilled water. It was then resuspended in distilled water and various amounts were injected intraperitoneally into mice. As in the case of the insoluble lipopolysaccharide reisolated from serum, this material also proved to be non-toxic with amounts up to 4000 µg. Its toxic activity was, however, restored by resolubilizing with EDTA.

These findings show that insoluble lipopolysaccharide preparations are non-toxic. Similarly, lipid A, obtained by acid hydrolysis, is insoluble in water and it had no lethal effect up to a dosage of 4000 µg. Lipid A from *S. minnesota* R345 solubilized by complexing with bovine serum albumin was then tested (lipid A: albumin, 1:0.5). As shown in Table 2, the complex was toxic. The LD_{50} corresponded to an amount of complex containing 1250 µg of lipid A. Albumin alone was found to be completely non-toxic.

The toxicity of three lipopolysaccharide preparations which were found to possess no anticomplementary activity has also been investigated. The prepa-

Table 2. Toxicity of various preparations used in this study

Lipopolysaccharide preparations	Dosage used					LD_{50} µg
	4000 µg	2000 µg	1000 µg	500 µg	250 µg	
Original <i>S. minnesota</i> R595 (soluble)	10	6	0	0	0	1750
Complement-treated R595 (insoluble)	0	0	0	0	0	>4000
Complement-treated R595 (solubilized with EDTA)	10	6	1	0	0	1750
Mg^{2+} and Ca^{2+} treated <i>S. minnesota</i> R595 (insoluble)	0	0	0	0	0	>4000
Mg^{2+} and Ca^{2+} treated R595 (solubilized with EDTA)	10	6	1	0	0	1750
<i>S. minnesota</i> R345 lipid A (complexed with albumin)	10	9	0	0	0	1250
<i>S. minnesota</i> R345 lipopolysaccharide	10	10	10	10	4	290
<i>S. minnesota</i> R60 lipopolysaccharide	10	10	10	7	4	320
<i>S. dysenteriae</i> R15 lipopolysaccharide	8	5	2	1	0	2000

rations were from *S. minnesota* R60, *S. minnesota* R345 and *S. godesberg* R15. As seen from Table 2, all three preparations are toxic.

DISCUSSION

In a recent communication [2] two *Salmonella* lipopolysaccharides have been compared with respect to their anticomplementary activity. One, derived from *S. minnesota* S form, contained lipid A and the complete polysaccharide region including the core and the O-specific chains [5], the other, derived from a *S. minnesota* R mutant (R595), contained only lipid A and 2-keto-3-deoxyoctonate (KDO), thus lacking the O-specific chains and the larger part of the core [5]. Both of these lipopolysaccharides were found to exhibit high activity of comparable order when assayed in the complement inactivation test. These results were confirmed in the present paper. However, it was now found that anticomplementary activity is not a property common to all lipopolysaccharides. A whole spectrum from high to negligible activity was obtained with lipopolysaccharides derived from various species, including S forms as well as R mutants. All the investigated lipopolysaccharides were water-soluble. Therefore, insolubility in water, which can be achieved with R lipopolysaccharides by precipitation with Mg^{2+} or Ca^{2+} ions and which results in the loss of both anticomplementary activity and toxicity, is not the reason for the different degrees in anticomplementary activity encountered among lipopolysaccharides of different origin. At present, no explanation can be offered for the fact that some lipopolysaccharides are anticomplementary and others are not. However, all lipopolysaccharides tested, regardless of their anticomplementary activity, proved to be toxic in the mouse lethality test. According to From *et al.* [25], lipopolysaccharides may utilize very little complement to exhibit endotoxic qualities, which may not rely on complement as the effective system. Thus decomplexation does not modify lipopolysaccharide-induced mortality.

Further findings, however, showed that the less active as well as the non-active preparations are potentially anticomplementary, but their activity is for some unknown reasons not expressed. All lipopolysaccharides contain a lipid component, the so-called lipid A [21], which can be liberated, in the form of a water-insoluble precipitate, from lipopolysaccharides by mild acid treatment. In this form, lipid A proved to be inactive when tested for anticomplementary and toxic properties. Also, lipid A made water-soluble by the aid of pyridine [20] or triethylamine [19] was found to be inactive in the anticomplementary test. However, water-soluble complexes formed from lipid A and water-soluble carriers, exhibited high anticomplementary activity.

Three different carrier preparations, which themselves did not inactivate complement, were studied: alkali-treated lipopolysaccharide, alkali-treated lipid A, and bovine serum albumin. According to the nature of the carrier, different quantities were necessary to bring a given amount of lipid A into an opalescent solution. Albumin was by far the best lipid A solubilizing agent and almost clear solutions were obtained with complexes containing lipid A and albumin in a ratio of 10:1. It was found that full anticomplementary activity was obtained only when the relative amount of carrier was sufficient to bring lipid A completely into solution.

With this technique, it was possible to demonstrate also that those lipopolysaccharides which themselves were not anticomplementary, contained a lipid A portion, which, after isolation and complexing, exhibited full activity. Thus, complexes composed of lipid A and treated lipopolysaccharide, both derived from the same inactive lipopolysaccharide, were highly active.

As in the case of the anticomplementary activity of lipid A, solubility is also necessary for toxic activity. While free, insoluble lipid A is non-toxic in mice, it becomes toxic after complex formation with albumin.

When previous [2,5] and present results had demonstrated that R form lipopolysaccharides containing only lipid A and KDO could exhibit both high anticomplementary and toxic activity, the question remained open still whether the sugar part of the molecule played a specific role in these reactions. The above results make it clear that KDO only functions as a solubilizing carrier and that it can be replaced by other carriers. Lipid A, therefore, is the active component of lipopolysaccharides in these reactions. The results also show that mere solubilization of lipid A by the aid of triethylamine [20] or pyridine [19] does not render lipid A anticomplementary. The results suggest that functional groups in lipid A must be exposed, which are blocked in aggregates of lipid A and which become available for interaction with complement in complexes with suitable carriers, where lipid A is present in a favourable conformation (see also [22,23]). A following paper will deal in more detail with the toxicity of lipid A carrier complexes.

It should be mentioned at this point that all lipopolysaccharides, including the alkali-treated preparations, show strong anticomplementary activity in the presence of specific rabbit antiserum when tested under otherwise identical conditions to those described in this paper. We have not studied the question of the degree to which natural antibodies against lipopolysaccharides, possibly present in the guinea pig serum, may play a role in the complement inactivation reaction (*cf.* [4]). Furthermore, the mechanism of complement inactivation and the

question as to which complement factors are involved in the reaction (cf. [3]) were not investigated.

Experiments, however, were performed to find out if the lipopolysaccharide molecule after interaction with complement (whole serum) had undergone chemical changes (cf. [2]). When lipopolysaccharide treated with pig serum was reisolated and purified, the resulting preparation was found to be neither toxic nor anticomplementary. No detectable qualitative or quantitative chemical changes in either the sugar or the fatty acid composition could be detected. The only significant change was the loss of solubility in water. Treatment of the reisolated lipopolysaccharide with EDTA completely restored solubility in water, toxicity and anticomplementary activity. These results are interpreted to mean that no chemical alterations occur on the lipopolysaccharide molecule after reaction with complement (serum). These results, however, do not exclude the possibility that physico-chemical changes regarding the membrane structure of lipopolysaccharide are effected by the interaction with complement.

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Biological Activities of Chemically Modified Endotoxins

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The cell wall glycolipid (somatic antigen) of the Re mutant of *Salmonella minnesota* is comparable in its endotoxic activity to the complete wild type lipopolysaccharide. The glycolipid is composed mainly of lipid A and 2-keto-3-deoxyoctonate (KDO); smaller amounts of ethanolamine and 4-aminoarabinose, and traces of amino acids are also present. The serological specificity is determined by KDO residues. In order to determine if non-lipid A constituents contribute to endotoxicity, amino groups and KDO units in the glycolipid were chemically modified by the introduction of substituents. When free amino groups were dinitrophenylated or succinylated the serological specificity was unaffected. By contrast, the original serological specificity was completely lost when the free hydroxyl groups of the KDO region were substituted with succinyl residues or the carboxyl groups converted to the methyl esters. In biological tests, however, the modified preparations exhibited full endotoxic activity. It is concluded that the polar groups, which determine the serological specificity of the KDO region of the glycolipid, do not play a specific role in endotoxicity.

The cell wall lipopolysaccharides (endotoxins, O antigens) of gram-negative bacteria are biologically highly active substances. Their injection into animals causes a variety of physio-pathological effects and evokes the production of specific antibodies [1-3]. Chemical studies over the past decade have provided sufficient information to establish a fairly detailed structure of lipopolysaccharides derived from *Salmonella*, *Escherichia coli*, *Shigella* and other genera [4,5]. They contain a polysaccharide portion, i.e. the O-specific chains and the core, and a lipid portion, called lipid A. Many attempts have been made to correlate biological activity to specific chemical structures of the macromolecule. It has been shown clearly that discrete structures in the polysaccharide portion of lipopolysaccharides carry the serological determinants responsible for antibody specificity [1,2,4]. The question of the existence of a defined region in lipopolysaccharides responsible for endotoxic activity is still under discussion, however. The recent finding that the incomplete lipopolysaccharides and glycolipids¹ of *Salmonella* R mutants, which lack O-specific chains and large parts of the core, are also potent endotoxins [6-8], has demonstrated that the long polysaccharide chain is not essential to endotoxicity. The most deficient

lipopolysaccharides, which have been obtained from R forms, are the glycolipids of *Salmonella* Re mutants. They contain lipid A, to which a trisaccharide of 2-keto-3-deoxyoctonate (KDO) is ketosidically bound [9,10]. Additional phosphate, ethanolamine, 4-aminoarabinose and trace amounts (about 0.1%) of amino acids are also present [10-12]. These glycolipids represent the simplest structure exhibiting endotoxic activity. It has been shown previously that KDO is the main serological determinant in these glycolipids [13].

The aim of the present investigation was to find out whether the KDO trisaccharide or other polar groups play a specific role in determining endotoxic activity of such a glycolipid. Therefore, without altering the structure of the lipid A part, functional groups in the polar region of the molecule were chemically modified. This resulted, in some cases, in changes of physico-chemical properties and in a complete loss of the original serological specificity. Biological assays performed with these modified preparations revealed, however, no changes in endotoxic activities compared to the genuine glycolipid. It is concluded that hydroxyl and carboxyl groups in the hydrophilic KDO region as well as amino groups are not specifically involved in endotoxic activity. Also, the serological reactivity of the sugar portion of the glycolipid is not associated with endotoxicity. These and other results [14,15] are in agreement with the hypothesis that the polysaccharide (oligosaccharide) portion of lipopolysaccharides (glycolipids) of the

¹ Unusual Abbreviation. KDO, 2-keto-3-deoxyoctonate.

¹ The term glycolipid was introduced as a designation of defective R mutant lipopolysaccharides which contain oligosaccharide units instead of polysaccharide, linked to lipid A [8].

cell wall of enterobacteria functions merely as a solubilizing "carrier" for the essential biologically active lipid A part.

MATERIALS AND METHODS

Bacteria and Glycolipid

The *S. minnesota* Re mutant R595 has been described previously [6]. The glycolipid was extracted by the phenol-chloroform-petroleum ether method [12].

Analytical Methods

Glucosamine was determined according to Strominger et al. [16], KDO by the thiobarbituric acid method [17], formaldehyde release after periodate oxidation with the chromotropic acid reagent [18], and heptose according to Dische [19] as modified by Osborn [20]. Free amino groups were estimated with 1-fluoro-2,4-dinitrobenzene as described by Ghuyssen and Strominger [21], ester determinations were carried out according to Snyder and Stephens [22] as modified by Tauber [23], phosphate according to Lowry et al. [24] and amino acids were analyzed in an amino acid analyzer (Beckman, model 120 B). Paper electrophoresis was performed according to Kichhöfen and Warth in a pyridine-acetic acid-water buffer (10:4:86, v/v/v) pH 5.4 [25]. For thin layer chromatography on cellulose of dinitrophenyl derivatives, a mixture of acetone-methanol-water (4:6:2, v/v/v) was used. Sugars were stained with the Trevelyan reagents, and substances with free amino groups with ninhydrin.

Chemical Modifications

Dinitrophenylation. 100 mg glycolipid was dissolved in 5 ml of water containing 0.8 ml of triethylamine. Then, 0.4 ml 1-fluoro-2,4-dinitrobenzene dissolved in 5 ml ethanol was added and the mixture stirred for 2 h at 20 °C. The addition of 80 ml ethanol resulted in a yellow precipitate (yield about 10 mg) which was removed by centrifugation. The supernatant solution was evaporated *in vacuo* to dryness and the residue washed three times with 100 ml acetone. A yellow residue, which gave a clear solution in water, was obtained (yield: 70 mg).

Succinylation in Water [26]. To 100 mg glycolipid suspended in 5 ml of 1.7 M sodium succinate, 500 mg of succinic anhydride was added in small portions over a 120 min period at 20 °C. The pH was maintained at 8.5 by the stepwise addition of 0.5 N NaOH. The resulting mixture was dialyzed against distilled water, neutralized with 0.1 N NaOH and lyophilized (yield: 80 mg).

Succinylation in Pyridine. A suspension of 100 mg glycolipid in 4 ml dried pyridine was heated to 100 °C for 1 min. After cooling to room temperature, 250 mg of succinic anhydride in 4 ml dried pyridine

were added at 20 °C. The mixture was treated for 30 sec in a boiling water bath and then for 3 h at 56 °C. After dialysis the clear solution was neutralized with 0.1 N NaOH and lyophilized (yield: 90 mg).

Esterification of Carboxyl Groups. 100 mg glycolipid was dialyzed at 4 °C for 3 days against 0.2 M EDTA (pH 7) and for a further 3 days against distilled water. The lyophilized material (99 mg) dissolved in 5 ml water was mixed under stirring with 50 ml methanol. To the suspension a freshly prepared solution of diazomethane in ether was added until the yellow color persisted. The clear solution was dried under reduced pressure, the residue suspended in water and lyophilized (yield: 85 mg).

Biological Tests

Mouse lethality tests were carried out at an environmental temperature of 37 °C [27,28]. The animals (NMRI strain female white mice weighing 16–20 g) were held for 4 h at 37 °C before injecting the samples. The preparations (serial dilutions in physiological saline or water) were injected intraperitoneally. Ten mice were used for each concentration and deaths were recorded after 24 and 48 h. LD₅₀ values were calculated according to the method of Reed and Muench [29].

Chick embryo lethality tests were carried out in the laboratory of Dr. A. Nowotny, Temple University, as described by Smith and Thomas [30]. Eleven days old chick embryos were injected intravenously with at least 4 different doses of endotoxin in 0.1 ml. For each concentration 30 chick embryos were used. The number of dead embryos in 24 h was recorded and the LD₅₀ calculated [29].

Pyrogenicity assays were performed in the laboratory of Drs. D. W. Watson and Y. B. Kim [31], University of Minnesota. The febrile response was determined in young adult American Dutch rabbits (1.0 to 1.2 kg), which were quartered in air-conditioned rooms at 23.9 °C. Each preparation was tested at 3 to 4 concentrations using 5 rabbits per concentration. Pyrogenicity is expressed as the minimal dose which causes a rise in temperature of 0.6 °C three hours after injection.

Irradiation protection experiments were carried out according to Langendorff et al. [32]. The preparations (10 µg in 0.3 ml physiological saline) were injected intraperitoneally into female white mice (100 animals per radiation dose) which were exposed 24 h later to X-irradiation (750 and 810 rad). The number of animals surviving 30 days after irradiation was taken as a measure of the protection activity of the preparations.

Hemagglutination inhibition and complement-activation tests were performed as described previously by Beckman et al. [33] and Galanos et al. [14], respectively.

Table 1. *Chemical and biological properties of unaltered and dinitrophenylated Re glycolipid*

The hemagglutinating system contained the original, alkali treated glycolipid, fixed to red blood cells, and the homologous antiserum. Recorded are the minimal concentrations of inhibitor which caused hemagglutination inhibition [33]. Anticomplementary activity is recorded as amounts of preparation which cause 50% inactivation of complement [14]. Mouse lethality tests were performed at 37 °C. The fatty acid content of the preparations was determined by gas liquid chromatography [36]

Glycolipid	Chemical properties					Biological activities		
	KDO	Glucosamine	Fatty acids	Amino acids	Amino groups	Hemaggl. inhib.	C' inactivation	Mouse lethality (LD ₅₀)
	%	%	%	%	μmoles/mg	μg/ml	μg	Chick embryo lethality (LD ₅₀)
Unaltered	17.0	12.6	50.0	0.12	0.3	8	4	15
Dinitrophenylated	18.0	15.0	51.3	0.03	0	2	5	20
								0.006
								< 0.001

RESULTS

Modification of Free Amino Groups

In preliminary experiments it was found that a 100% conversion of amino groups to hydroxyl groups with nitrous acid [34] or to *N*-acetyl groups with acetic anhydride [35] could not be achieved. Under various conditions, not more than 60 to 75% of the free amino groups in the glycolipid were modified. In biological assays, the modified preparations exhibited full activity in hemagglutination inhibition, anticomplementary, and mouse lethality tests.

Dinitrophenylation on the other hand gave complete substitution of the free amino groups in the glycolipid. In hydrolysates (6 N HCl, 100 °C, 5 h) of the soluble modified preparation, no ethanolamine or phosphorylethanolamine could be detected by paper electrophoresis indicating that the amino groups were quantitatively substituted. On thin layer chromatography of the hydrolysates, two yellow spots were visible with *R_F* values corresponding to those of dinitrophenylated ethanolamine and phosphorylethanolamine, respectively. Sugar analysis revealed that the KDO and glucosamine content in the modified preparation was unchanged (Table 1). Similarly, no qualitative or quantitative changes in the fatty acid composition were detectable. On the other hand, about 75% of the amino acids present in the original glycolipid (0.12%) were removed in the course of purification of the modified preparation. This material was recovered in the insoluble precipitate described in Material and Methods.

Table 1 summarizes the results of biological assays performed comparatively with the original and dinitrophenylated glycolipid. In all tests the modified preparation proved to be highly active. As an inhibitor of passive hemagglutination and in the chick embryo test, it seems to be even more active than the original glycolipid. This is probably due to its better solubility in water.

Complete substitution of the amino groups in the glycolipid was also achieved by succinylation with succinic anhydride in aqueous medium according to McIntire *et al.* [26]. A few hydroxyl groups (1.2 moles

per 3 KDO) were also succinylated by this procedure (Table 2). The data given in Table 2 show that serological specificity and anticomplementary activity of the modified preparation were unchanged and that it exhibited slightly higher activity in the mouse lethality, pyrogenicity and irradiation protection tests compared with the original glycolipid.

Modification of Hydroxyl and Amino Groups

In order to substitute both amino and hydroxyl groups of the glycolipid, succinylation with succinic anhydride was carried out in pyridine, in which both reactants and the reaction products are soluble. After preliminary experiments performed under various conditions, succinylation was carried out in pyridine at 56 °C for 3 h (see also [43]).

The degree of substitution of hydroxyl groups with succinyl groups was calculated from *O*-ester determinations performed on the original and modified glycolipids, which contain a large proportion of long chain fatty acid ester groups in the lipid A part (see [5]). The substitution of amino groups was determined with fluorodinitrobenzene.

The modified preparation thus obtained contained about 8 moles of succinic acid per 3 moles KDO (Table 2), i.e. 8 out of 11 hydroxyl groups, available in the KDO trisaccharide and two 3-hydroxymyristic acid residues in lipid A [36], were substituted. In addition, all amino groups were substituted. The product, which was water-soluble, proved to be highly resistant to periodate oxidation. Only traces of formaldehyde were formed and in the thio-barbituric acid determination, only 20% of the KDO was detectable. When this preparation was assayed in the hemagglutination test, it was found that the serological specificity of the preparation was completely altered. Even with 250 μg no inhibition of passive hemagglutination was seen in the parent glycolipid anti-glycolipid serological system (see Table 2). This is in agreement with previous findings [13] that KDO is an immunodominant structure in *S. minnesota* R595 glycolipid. Furthermore, it could be shown that serological

Table 2. *Chemical and biological properties of the unaltered and succinylated Re glycolipid*
In the irradiation protection test, out of 100 animals, which were not pretreated with endotoxin, 8 (with 750 rad) and 4 (with 810 rad) survived. n.d. = not done

Glycolipid	Chemical properties					Biological activities						
	KDO	Glucosamine	Amino groups	Ester		Hemagglutination inhibition		C' inactivation	Mouse lethality (LD ₅₀)	Pyrogenicity	Irradiation protection	
				present	introduced	before	after				750	810 rad
						alkali treatment						
Unaltered	μmoles/mg	%	μmoles/mg	moles/3 KDO	μg/ml		μg	μg	μg/kg	% surviving		
	0.67	12.6	0.3	1.16	0	2	2	4	15	0.0035	50	2
Succinylated	0.63	12.6	0	1.51	1.2	2	n.d.	3	5	0.0013	64	14
in water	(0.13)	9.5	0	3.01	8.1	> 250	16	22	12	0.0020	60	6
in pyridine												

Table 3. *Chemical and biological properties of the Re glycolipid and its methylester*

Glycolipid	Chemical properties			Biological activities			
	KDO	Glucosamine	P	Hemagglutination inhibition		C' inactivation	Mouse lethality (LD ₅₀)
				before	after		
				alkali treatment			
	%	%	%	μg/ml	μg	μg	
Unaltered	16.0	12.6	2.6	4	4	4	15
Methylester	(10.5)	11.0	2.3	> 250	8	5	80

specificity could be restored by treatment of the succinylated preparation with NaOH (0.25 N, 56 °C, 1 h). In spite of the complete loss of the original serological specificity, the succinylated preparation exhibited full lethal toxicity, pyrogenicity, and irradiation protection (Table 2). Only a slight decrease in the ability to inactivate complement was observed (see also [43]).

Modification of Carboxyl Groups

Esterification with diazomethane of the free carboxyl groups of KDO resulted in a preparation which was less soluble in water than the parent glycolipid. The esterified preparation proved to be much more resistant to acetic acid hydrolysis (pH 3.4) compared with the original glycolipid, which is easily cleaved under these conditions to yield free KDO and lipid A. Neither free KDO nor its methyl ester could be detected in the acetic acid hydrolysate of the modified glycolipid by paper electrophoresis. After sulfuric acid hydrolysis (0.25 N, 100 °C, 8 min) about 60% of the KDO, detectable in the parent glycolipid, became available for the thiobarbituric acid assay. However, after saponification of the modified glycolipid (0.25 N NaOH, 56 °C, 1 h) all KDO residues could be liberated by acetic acid and were detectable with thiobarbituric acid. Neither the

phosphate nor the glucosamine contents were changed in the modified preparation (Table 3). The amount of methyl ester groups was not determined, but the properties of the modified preparation indicate a high degree of substitution (it can be assumed that phosphate groups were also substituted).

Table 3 summarizes the results of the biological assays. Hemagglutination inhibition tests showed that the serological specificity of the parent glycolipid was completely lost after esterification, as 250 μg of the modified glycolipid did not inhibit antibodies against the parent glycolipid. That this was entirely due to the modification of the carboxyl groups could be demonstrated by saponification of the ester groups. After alkali treatment of the modified glycolipid, full serological specificity was restored. In the complement inactivation test, the modified preparation showed full activity. A certain decrease in lethal toxicity of the esterified glycolipid was detected, probably due to its lower degree of solubility in water.

DISCUSSION

The lipopolysaccharides of Enterobacteriaceae are potent endotoxins [1-3]. Chemical studies have revealed that the polysaccharide components of lipopolysaccharides derived from different species

vary widely with respect to composition and structure [4,37]. On the other hand, the lipid component, designated lipid A, is structurally very similar in these lipopolysaccharides [4,38]. It was therefore postulated [38,39] that lipid A was essential or responsible for the various endotoxic activities. Many attempts were made to show that free lipid A, isolated by hydrolysis of lipopolysaccharides, exhibits biological activity and, in fact, active preparations have been described [39]. However, even the most active lipid A preparations thus obtained were less toxic on a weight basis than the parent lipopolysaccharides (see [4,39]) (and not of higher activity, as should have been expected). Furthermore, the results could not be reproduced in some other laboratories (see [40]). The question of whether lipid A represents the biologically active principle in endotoxic lipopolysaccharides thus remained open.

An important step forward was made when defective lipopolysaccharides of various R mutants were investigated. It could be demonstrated that even the most defective ones, i.e. the glycolipids of Re mutants, which contain lipid A and KDO only, are also potent endotoxins [6-8]. These results showed unequivocally that the biologically active structure of lipopolysaccharides comprises the KDO/lipid A region.

In order to define the active center more closely three lines of investigation were pursued: (a) details of the structure of lipid A were evaluated, especially the nature and distribution of the long chain fatty acids [9,36]; (b) the toxicity of free isolated lipid A was reinvestigated under various conditions [14,15], and (c) polar groups in a Re mutant glycolipid were chemically modified in order to find out if KDO, ethanolamine, or other non-lipid A constituents contribute specifically to endotoxicity. The results of these latter investigations are presented in this paper.

The glycolipid of Re mutants contains lipid A and a KDO trisaccharide, which is partly substituted by a phosphorylethanolamine group [10]. Additional ethanolamine, 4-aminoarabinose [11], and trace amounts of amino acids (peptides) [12], linked in an unknown way, are also present. The serological specificity of the glycolipid is determined by KDO residues [13]. Wild type lipopolysaccharides contain the same Re glycolipid as an underlying structure, together with further sugars residues of the core and O-specific chains [4]. In all these preparations, therefore, free amino groups (ethanolamine, amino acids), carboxyl groups (KDO), and hydroxyl groups (KDO and other sugars) are present. The polar groups in the glycolipid of the Re mutant of *S. minnesota* R595 were chemically modified by the introduction of substituents under conditions where no gross alterations in the lipid part could be expected to occur.

Amino groups were dinitrophenylated and succinylated. Both reactions resulted in a quantitative

substitution without alteration of the basic structure of the molecule. In contrast to the original glycolipid, the modified preparations were completely water-soluble.

In biological tests, products with modified amino groups exhibited activities comparable to, or even higher than, those of the parent glycolipid. These results are in agreement with the findings of McIntire *et al.*, who have studied dinitrophenylated S form lipopolysaccharides [41].

It should be mentioned that identical results were obtained with dinitrophenylated endotoxins derived from a number of other R mutants. In all cases, a main water-soluble dinitrophenylated fraction was obtained, which exhibited full endotoxic activity. From these preparations, as much as 90% of the amino acids, originally present in the glycolipid, was absent. This suggests that peptides are not involved in endotoxicity. However, when S form lipopolysaccharides were dinitrophenylated, about equal amounts of a water-soluble and a water-insoluble fraction were obtained. In the soluble preparations, which exhibited high biological activities, the bulk of the core constituents and lipid A were present. In contrast, the insoluble fractions, which were biologically inactive (probably due to their insolubility) contained mainly the O-specific sugars and most of the peptide material. At present, no explanation can be given for the mechanism underlying this kind of fractionation, which has been encountered hitherto only under the conditions of dinitrophenylation.

Hydroxyl groups and amino groups in the glycolipid were modified by succinylation in pyridine. The reaction led to a substitution of 8 out of the 11 hydroxyl groups, which are available in the KDO trisaccharide [10] and two 3-hydroxymyristic acid residues [36] of lipid A. In addition, all amino groups were substituted. The preparation was clearly soluble in water. Inhibition studies revealed that the modified preparation was completely devoid of the original serological specificity. This is in agreement with the previous observation, that KDO residues represent immuno-specific structures in the parent glycolipid [13]. Treatment of the product with alkali cleaved the substituents and, simultaneously, restored serological specificity. The modified preparation showed reduced activity in the complement inactivation test, probably due to its water-solubility (absence of aggregation), but as an endotoxin (lethality, pyrogenicity, irradiation protection) it was at least as active as the parent glycolipid (see also [43]).

Esterification of the carboxyl groups of KDO in the Re glycolipid yielded a preparation of low solubility and altered chemical reactivity. The absence of free carboxyl groups in the molecule resulted in a marked resistance to acid hydrolysis: in contrast to glycolipids and lipopolysaccharides, the ketosidic linkages of the substituted KDO residues were not

cleaved on heating at pH 3.4. As in the case of the succinylation of hydroxyl groups of the KDO residues, esterification of their carboxyl groups also completely masked the original serological specificity. Both acid lability and serological specificity could, however, be restored on alkaline saponification. On the other hand, the modified product proved to be as active or only slightly less active than the parent glycolipid, when tested for complement inactivation and mouse lethality.

From these results it is concluded that the polar groups in the core region of lipopolysaccharides do not play a specific role in determining endotoxicity. Since the serological specificity of some of the preparations had been altered drastically without resulting in significant changes in biological activity, it is obvious that immunological determinants as represented by KDO or other sugar residues in the polysaccharide part of lipopolysaccharides are not directly involved in the manifestation of endotoxic reactions.

It is generally accepted that mouse lethality and, even more so, pyrogenicity [31] and chick embryo lethality [3] are suitable tests for determining acute endotoxic activity. In addition, irradiation protection [32], was assayed for some preparations as an example of the "delayed type" activities of endotoxins. It is known, however, that these activities can be dissociated from the acute type activities, and it was found, for instance, that the water-soluble dinitrophenylated preparations were not active in eliciting the Shwartzman reaction (kindly tested by Dr. A. Nowotny). Similarly, it is known that the ability of lipopolysaccharides to inactivate complement does not always parallel their endotoxic activity [14]. Such examples were also encountered in this study: some *N*-acetylated or succinylated preparations exhibited decreased activity in the complement test but were fully active as endotoxins.

For many endotoxic properties, the physicochemical state of the preparation, (such as water solubility or degree of aggregation) may be crucial. Changes in these properties sometimes resulted in increased activity and sometimes in decreased activity (see Tables 1 and 3).

Such a dependence of endotoxic activity on physical state has been found previously, when the glycolipid of the Re mutant of *S. minnesota* was compared with the parent S form lipopolysaccharide [42]. The glycolipid exhibited a lower toxicity (mouse lethality, pyrogenicity). However, when the glycolipid was tested in the presence of triethylamine [42], which causes disaggregation, its activity was significantly increased being equal (lethality) or even higher (pyrogenicity) compared with the S form lipopolysaccharide. Similarly, dinitrophenylation or mild succinylation, which solubilizes (disaggregates) the

glycolipid, seems to have an analogous effect on its biological activity (see Table 1).

In summary, the results presented in this paper show that the KDO part of Re glycolipids, and hence the polysaccharide portion of lipopolysaccharides, is not specifically involved in endotoxic activities. It will be shown more directly in a subsequent paper [15] that the hydrophilic part of lipopolysaccharides functions merely as a solubilizing carrier for the water-insoluble lipid A component, which itself represents the biologically active principle in endotoxic bacterial lipopolysaccharides.

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VI

Biological Activities of Lipid A Complexed with Bovine-Serum Albumin

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Lipid A preparations from the lipopolysaccharides of four *Salmonella minnesota* R mutants and one *Escherichia coli* 0100 R mutant were assayed as soluble complexes with bovine serum albumin for lethality in mice, pyrogenicity in rabbits and complement inactivation. Although generally less active than endotoxic lipopolysaccharides, these lipid-albumin complexes nevertheless exhibited strong biological activity. These results indicate that lipid A contains the endotoxic principle of bacterial lipopolysaccharides.

The lipopolysaccharides (endotoxins) of gram-negative bacteria (*Salmonella*, *Shigella* and *Escherichia*) consist of a specific polysaccharide covalently linked to a common lipid termed lipid A [1]. Attempts have been made to define which of these two components determines the biological activity of these molecules by separating the two moieties by acid hydrolysis and testing them individually in different biological systems. In such experiments the (degraded) polysaccharides have always been non-toxic [2] whereas conflicting results have been obtained with lipid A. Some investigators find that their lipid A preparations give rise to pyrogenicity [3-5], tumor inhibition [6], tumor necrosis [7], enhanced dermal reactivity to epinephrine [8], non-specific protection [4,9,10], interferon release [11] and adjuvanticity [4,12] whereas others find that their preparations do not enhance dermal reactivity to epinephrine [13,14] and are unable to induce fever and tumor necrosis [15,16]. These discrepancies have been assumed to be due to differing degrees of degradation or insolubility of the lipid A preparations. In order to obtain soluble lipid A, solubilizing agents such as Tween were used or, alternatively, the lipopolysaccharide was cleaved in the presence of casein so that a soluble lipid-protein complex (lipo-casein) was obtained. Such preparations exhibited strong pyrogenicity [6,17-19] but it was difficult to exclude the possibility that this activity was not due to the presence of small amounts of unaltered lipopolysaccharide.

Abbreviations. KDO, 2-keto-3-deoxyoctonate; lipid-albumin, complex of lipid A and bovine serum albumin.

A new approach to this problem became possible with the observation that *Salmonella* R mutants synthesise lipopolysaccharides (glycolipids) lacking O-specific side chains and large parts of the core [20]. In particular, the finding that the glycolipids from Re mutants which contain only lipid A and 2-keto-3-deoxyoctonate (KDO) were potent endotoxins [20-22], showed that the polysaccharide portion of the molecule was not essential to endotoxicity.

More direct evidence of the role of lipid A in endotoxicity was obtained [23] by testing the activity of free lipid A that had been solubilized by complexing with protein carriers. In particular, complexes of lipid A and bovine serum albumin were found to have strong anticomplementary activity and toxicity for mice. The present paper describes and reviews the endotoxic properties of those complexes which have been studied recently in this and other laboratories.

MATERIALS AND METHODS

Bacteria and Lipopolysaccharides

The R form strains *Salmonella minnesota* R5 and R595 were obtained from J. Schlosshardt (Zentral-laboratorium für bakterielle Darminfektionen, Potsdam, DDR) [20]. *S. minnesota* R345 and R60 from F. Kauffmann (Statens Seruminstitut, Copenhagen, Denmark) [20] and the *Escherichia coli* strain EH100 from P. H. Mäkelä (State Serum Institute, Helsinki, Finland) [24]. Bacteria were cultivated [25] and extracted with phenol-chloroform-petroleum ether [26].

*Lipid A and Complexes of
Lipid A and Bovine-Serum Albumin*

Lipid A was prepared from R form lipopolysaccharides or glycolipids by acetic acid hydrolysis (0.1 N, 2.5 h, 100 °C) as described previously [23]. Absence of 2-keto-3-deoxyoctonic acid (KDO) and heptose was taken as a criterion for purity. The complexing procedure of lipid A with bovine serum albumin has also been described previously [23]. Briefly, lipid A (10 mg) in water (5 ml) was solubilized by addition of triethylamine (5 µl). This solution was mixed with a solution of bovine serum albumin (10 mg) in water (5 ml). The resulting mixture was dried in a rotary evaporator under reduced pressure, whereby triethylamine was removed and complexing was allowed to take place. The complex was redissolved in distilled water and lyophilized. These lipid-albumin complexes, like R form lipopolysaccharides, tend to precipitate in the presence of salts. Therefore, stock solutions were prepared in distilled water and kept frozen. Small amounts of precipitate formed sometimes after thawing were redissolved by sonication. Appropriate amounts of the stock solutions were mixed with saline, or if necessary with buffer, just before the experiment.

Analytical Methods

KDO was determined by the thiobarbituric acid method [27] and heptose according to Dische [28].

Biological Tests

Mouse lethality tests were performed as described earlier [23]. The LD₅₀ was calculated according to Reed and Muench [29].

Pyrogenicity assays were carried out as described previously [3]. The febrile response in rabbits is expressed as the minimal dose which causes a rise in temperature of 0.6 °C 3 h after injection (MPD-3).

The biological activity of the complexes was calculated on the basis of their lipid A content.

RESULTS

Lipid A was isolated from the lipopolysaccharides (glycolipids) of four R mutants of *Salmonella minnesota* (R60, R345, R5 and R595) and one R mutant of *E. coli* 0100 (EH100). The respective lipid A preparations were complexed with bovine serum albumin in a 1:1 ratio and the complexes were tested comparatively with the corresponding lipopolysaccharides for lethal toxicity and pyrogenicity. Table 1 summarizes the results. In mouse lethality tests the complexes containing lipid A from *S. minnesota* R345, R60 and *E. coli* EH100 were found to be of comparable activity (LD₅₀ = 1.7 mg). This activity is of the same order as that of the glycolipid from *S. minnesota* R595 (LD₅₀ = 1.7 mg) but lower than that of the other parent lipopolysaccharides. The complexes containing lipid A from *S. minnesota* R595 and R5 showed a higher LD₅₀ value (about 4 mg). Lipid A not complexed with albumin and albumin alone were not toxic in the doses tested.

In pyrogenicity assays the lipid A complexes exhibited high activity (Table 1). The minimal pyrogenic dose for all preparations was found to be between 0.003 and 0.013 µg/kg, i.e. of the same order as it was found for the glycolipid of strain *S. minnesota* R595 and other endotoxins [3, 21].

Table 1 also contains results of complement inactivation tests, which have in part been published

Table 1. *Biological properties of lipid-albumin complexes as compared with the parent lipopolysaccharides (glycolipids)*
LPS = lipopolysaccharide; complex = lipid-albumin complex; n.d. = not determined

Lipopolysaccharide or lipid-albumin complex		Lethality for mice LD ₅₀	Pyrogenicity MPD-3 µg/kg	Bone marrow reaction ED ₅₀ µg	Complement inactivation with 10 µg preparation %	Limulus lysate gelation (solid gel) [31] µg/ml
<i>S. minnesota</i>	R60	LPS 300	n.d.	n.d.	30 ^a	n.d.
		complex 1700	0.003	18.8	86	n.d.
	R345	LPS 300	n.d.	6.2	5 ^a	n.d.
		complex 1700	0.013	7.2	85	10 ⁻⁸
	R5	LPS 650	n.d.	n.d.	70	10 ⁻¹²
		complex 4000	0.007	10.9	85	n.d.
	R595	LPS 1750	0.004	3.9	80	10 ⁻¹³
		complex 4000	0.007	11.0	86	n.d.
<i>E. coli</i> EH100	LPS	n.d.	n.d.	n.d.	15 ^a	n.d.
	complex	1700	n.d.	n.d.	80	n.d.
Albumin control		> 10000	> 100	> 100	0	> 10 ⁻³

^a Higher amounts of these preparations do not lead to inactivation of more complement; in fact, lower amounts are inactivated.

previously [23]. The activity of the lipid · albumin complexes, expressed as the percentage inactivation obtained with 10 µg of the preparation, was found to be similar for all complexes tested (about 85%), and similar to the glycolipids of *S. minnesota* R595 and R5. The lipopolysaccharides of *S. minnesota* R345, *S. minnesota* R60 and *E. coli* EH100 were much less active, and here, the small activity is probably due to natural antibody-mediated complement interaction [23].

DISCUSSION

Recent studies [23] have shown that the lipid A components of *Salmonella* lipopolysaccharides have high anticomplementary activity when solubilized by certain protein carriers. One lipid A preparation (from *S. minnesota* R345) also exhibited lethal toxicity in mice when complexed with bovine serum albumin. These studies have now been extended to complexes containing lipid A preparations from four *S. minnesota* R mutants and one *E. coli* R mutant.

The results presented in this paper show, that all these lipid · albumin complexes when tested in rabbits are potent pyrogens and are almost as active as the corresponding lipopolysaccharides (glycolipids). The complexes showed typical biphasic fever curves.

Lethal toxicity tests were performed in mice. Three of the five lipid A preparations (R60, R345, EH100) were comparable in activity with the *S. minnesota* R595 glycolipid. Two were less active (R595, R5). The reason for these differences is not known.

Some lipid · albumin complexes have been tested for biological activity in other laboratories. Yoshida *et al.* [30] using the bone marrow reaction showed that all complexes tested were highly active, the activity being in the same order as that of S or R form lipopolysaccharides (see Table 1). Yin *et al.* [31] have studied the activity of lipid · albumin complexes in inducing gelation of limulus lysate. In this test the complex was significantly less active than the parent glycolipid. Alexander and Evans [32] found that a lipid · albumin complex (lipid A from *S. minnesota* R345) renders macrophages from the peritoneal cavity of mice cytotoxic to other mouse cells. The complex was found to be as active as *S. minnesota* R345 lipopolysaccharide.

The results presented and discussed in this paper show that lipid A represents the active component in lipopolysaccharides responsible for endotoxic activities (pyrogenicity, lethal toxicity for mice, bone marrow reaction) and some other biological effects elicited by endotoxins (complement inactivation, macrophage activation).

Furthermore, the structures necessary for the biological activity of lipid A are not significantly altered by acid hydrolysis during the isolation procedure. Some degradation may account for the lower activity in certain tests. Moreover, physicochemical factors such as the solubility and state of aggregation of the molecules may play a role in endotoxicity [33]. In free lipid A, for example, active structures may be masked by a high degree of aggregation [35]. The exposure of a "toxic conformation" may be induced by the polar polysaccharide moiety (as in lipopolysaccharides) [34] or by a protein carrier such as bovine serum albumin. Thus the present evidence suggests that the polysaccharide component plays no primary role in the endotoxicity of lipopolysaccharides though it may be important for the full expression of lipid A activity by stabilising the "toxic conformation". However, the endotoxic and related biological activities of lipopolysaccharides are essential properties of the lipid A component.

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VII

Preparation and Properties of Antisera against the Lipid-A Component of Bacterial Lipopolysaccharides

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The lipid-A component of lipopolysaccharides, when presented in a suitable form, acts as an immunogen in rabbits, giving rise to the production of circulating antibodies. Highest anti-lipid-A titers were obtained on immunization with acid-treated bacterial cells coated with lipid A. Anti-lipid-A activity of the immune sera could be assayed by passive hemolysis of lipid-A-sensitized erythrocytes in the presence of complement. On filtration of the immune globulins on Sephadex G-200, antibodies against lipid A were detected in the IgM and IgG fractions. Lipid A preparations derived from various *Salmonella* and one *Escherichia coli* strain exhibited strong serological cross reactions with anti-lipid-A antisera. Furthermore, lipid-A antiserum also cross-reacted with all lipopolysaccharides tested, although in some cases to a low degree. On the other hand, only 8 out of 13 antibacterial antisera interacted with lipid-A-coated red cells. Finally, it was found that anti-lipid-A antibodies are effective in sensitizing *E. coli* O111 bacteria for intraperitoneal phagocytosis.

The lipopolysaccharides of Enterobacteriaceae are constituents of the outer cell wall [1]. They represent the endotoxins and the somatic antigens of the bacteria. Chemically, the lipopolysaccharides of wild-type strains are macromolecules made up of two distinct regions of contrasting character, a hydrophilic polar region, the polysaccharide, consisting of the O-specific chains and the basal core [1,2], and a hydrophobic lipid portion, the lipid A [3]. R mutant strains contain defective lipopolysaccharides; the O-specific chains are absent and the core polysaccharide depending on the site of mutation is present in a more or less complete form [1,2].

Immunization of animals with bacteria or isolated lipopolysaccharides leads to the production of specific anti-lipopolysaccharide antibodies. They are directed mainly against determinants in the sugar region of the molecule [1]. Circulating antibodies with anti-lipid A specificity have not yet been identified.

The present results show that under certain conditions lipid A acts as an immunogen in rabbits, giving rise to the production of antibodies which react specifically with lipid A. Some properties of anti lipid-A antibodies were evaluated and their possible biological significance is discussed.

MATERIALS AND METHODS

Bacteria

In general, bacteria were cultivated in a fermenter as described previously [4]. They were killed by ad-

dition of phenol to the culture (10 g/l), washed twice with distilled water, twice with acetone and once with ether, and dried *in vacuo*. They were stored at room temperature.

In some cases, as mentioned in the text, freshly heat-killed bacteria were used. They had been cultivated on agar [5] and heated at 100 °C for 2 h.

Lipopolysaccharides, Lipid A, and Alkali-Treated Lipid A

Lipopolysaccharides were extracted from smooth-form bacteria by the phenol/water method [6] and from rough-form bacteria by the phenol/chloroform/petroleum ether method [7].

Free lipid A was obtained by hydrolysis of the respective lipopolysaccharide in 1% acetic acid at 100 °C for 2 h [8,9]. The water-insoluble free lipid A was washed several times with distilled water and dried *in vacuo*. No 2-keto-3-deoxyoctonate could be detected [10] in such preparations. Lipid A was solubilized by addition of triethylamine (1 µl) to a suspension of lipid A in water (2 mg in 2 ml) [11].

Alkali-treated lipid A was prepared by heating free lipid A in 0.25 N NaOH at 56 °C for 1 h (see [12]). After removal of insoluble material the supernatant was neutralized with acetic acid, and, after removal of the precipitate formed, dialyzed against distilled water. Precipitated material was again removed by centrifugation and the supernatant, containing soluble alkali-treated lipid A, was freeze-dried.

Acid-Treated Bacteria Coated with Lipid A

For hydrolysis, the dried bacteria (mR 595) were washed twice with 1% acetic acid, then suspended in 1% acetic acid (1 g/50 ml) and heated at 100 °C for 2 h. They were washed with distilled water and dried *in vacuo*. This treatment was meant to remove the 2-oxo-3-deoxyoctonate part of the glycolipid.

Lipid A was suspended in distilled water (1 mg/ml) and solubilized by addition of triethylamine (0.5 µl/ml). Aliquots of this solution were mixed with 2 ml of a suspension of hydrolyzed bacteria in distilled water (1 mg/2 ml), and the mixtures dried in a rotary evaporator. Acid-treated bacteria coated with 10, 100, or 500 µg lipid A per 1 mg dry weight were prepared in this way.

Similarly, non-hydrolyzed bacteria were coated with lipid A.

Immunization Schemes

Anti-bacterial sera against heat-killed S and R form bacteria were prepared as described previously [5].

For the production of anti-lipid-A antisera, two different immunization schemes were used. In the first, New Zealand white rabbits (2–2.5 kg) received four intravenous injections of the antigens on days 1 (100 µg), 4 (200 µg), 7 (300 µg), and 11 (500 µg), respectively. The animals were bled on day 16 by cardiac puncture.

In the second scheme, silver champagne rabbits were used (1.5 kg). The antigen suspension (1.5 mg in 1 ml water) was mixed with an equal volume of incomplete Freund's adjuvant. Of this suspension 1 ml was injected subcutaneously at 4 different sites. Two immunizations seven weeks apart were carried out. Twenty days later, blood was taken from the ear vein. All sera were decanted by heating (30 min, 56 °C).

The following preparations were used as antigens: hydrolyzed and non-hydrolyzed dried bacteria both untreated and coated with lipid A, freshly heat-killed bacteria, and incomplete Freund's adjuvant alone. The ratio of bacteria to lipid A was 100:1 (w/w) for the first, and 2:1 (w/w) for the second scheme.

Passive Hemolysis Test

It was found that the passive hemolysis test (compare [12], [13]) represents a convenient and sensitive method for determining anti-lipid-A antibodies. The test was carried out in large hemagglutination titer plates (well diameter 15 mm, capacity 1 ml), using veronal buffer (pH 7.8) as diluent. Human erythrocytes of blood group B Rh⁺ were sensitized (37 °C for 30 min) with triethylamine-solubilized lipid A or alkali-treated lipid A (60 µg/0.2 ml packed cells).

In cross reaction experiments, red cells were coated with S form or alkali-treated R form lipopolysaccharides (120 µg/0.2 ml packed cells). To each well, containing the serum dilution (0.2 ml) and the suspension (0.5%) of the sensitized erythrocytes (0.2 ml), complement was added (25 µl guinea pig serum). The mixtures were incubated (37 °C for 30 min) and hemolysis recorded.

Absorption and Desorption of Anti-Lipid-A Antibodies

Anti-lipid-A antiserum (1 ml) was mixed with packed formalinized red cells (0.1 ml) which had been sensitized with alkali-treated lipid A (100 µg). The mixture was stirred at room temperature (15 min) and the cells separated by centrifugation.

For desorption of the anti-lipid-A antibodies [14] the cells after absorption were stirred in 0.1 M glycine-HCl buffer (pH 2.2, 2 ml) at room temperature (1 h). After centrifugation, the supernatant was neutralized with NaOH and concentrated by ultrafiltration to the original volume (1 ml).

Chromatographic Separation of Immunoglobulins [15]

Antiserum (20 ml) was diluted with saline (40 ml) and precipitated with ammonium sulfate (60 ml, 4 M, pH 7.0) at 4 °C. The precipitate was dissolved in saline (10 ml) and dialyzed against 1 l phosphate-buffered saline (0.01 M phosphate, 0.14 M NaCl, pH 7.3). The immunoglobulin-containing fraction was placed on the bottom of a Sephadex G-200 column (5.0 × 100 cm) equilibrated with Tris-glycine buffer (0.2 M Tris, 0.5 M glycine adjusted with conc. HCl to pH 7.6, with butanol added to a volume fraction of 0.02). Then, 20 ml Tris-glycine buffer (pH 7.6) containing 20% glucose were applied to the column and elution was carried out ascendingly with Tris-glycine buffer (pH 7.6) at a flow rate of 16 ml/h. Fractions of 8 ml were collected.

Phagocytosis Test

The procedure described by Whitby and Rowley [16] was used with slight modifications. An overnight culture of *Escherichia coli* 0111 was diluted with meat extract medium to a concentration of about 5×10^5 cells/ml. The cell suspension (0.5 ml), appropriate serum dilutions (0.5 ml) and meat extract medium (4 ml) were incubated (37 °C, 30 min) and then placed in an ice bath. From each sample, 0.2 ml were injected intraperitoneally into each of two mice. At various time intervals cold saline (2 ml) was injected intraperitoneally and the animals killed immediately. 1 ml fluid was removed from each animal and 0.1 ml was plated on agar plates in duplicate. After overnight incubation (37 °C) the number of colonies was counted.

RESULTS

*Determination
of Anti-Lipid-A Antibodies*

In the past, passive hemagglutination and hemolysis have been used successfully for the identification of anti-O-antibodies [12,13,17]. Preliminary experiments had shown that passive hemolysis can also be adopted as a sensitive test for determining anti-lipid-A antibodies. Human erythrocytes of blood group B Rh⁺ were used since they did not cross-react with normal rabbit sera or with anti-lipid-A antisera. It was found that they can be sensitized equally well with triethylamine-solubilized lipid A [11] or with alkali-treated lipid A. Like lipopolysaccharides [12,13,17], both these preparations coat erythrocytes spontaneously. Erythrocytes sensitized with either lipid A or alkali-treated lipid A led to essentially identical results. For convenience, alkali-treated lipid A was generally used for coating the erythrocytes.

The passive hemagglutination test was not employed in these studies because lipid-A-sensitized cells tend to aggregate non-specifically in the presence of serum. Another specific assay for anti-lipid-A activity involving liposomal model membranes is described in the following paper [31].

*Anti-Lipid-A Activity
of Various Immune Sera*

Rabbits were immunized with various antigens according to the schemes described in Materials and Methods. The bacteria used were *Salmonella minnesota* R595 [2]. The lipid A preparation was derived from the lipopolysaccharide of *Salmonella minnesota* R345 [2]. For determining anti-lipid-A activity in the respective sera, the passive hemolysis test was applied, using red blood cells coated with alkali-treated lipid A from *S. minnesota* R345. In general, subcutaneous immunization in incomplete Freund's adjuvant led to a slightly higher anti-lipid-A titer compared with the intravenous immunization scheme. With both schemes, best results were obtained when hydrolyzed *S. minnesota* R595 bacteria, coated with lipid A, were used as antigen. A significant response was also obtained by immunizing with acid-treated bacteria, or with non-hydrolyzed bacteria coated with lipid A. Immunization with either phenol-killed or heat-killed bacteria led to low levels of anti-lipid-A activity. No antibodies could be detected in the pre-immunization sera or after injection of incomplete Freund's adjuvant. Some of the results are summarized in Table 1.

One rabbit was immunized intravenously with hydrolyzed bacteria (*S. minnesota* R595) coated with a lipid-A preparation obtained from the lipopolysaccharide of an *E. coli* strain (*E. coli* EH100 [18]). The test system for determining anti-lipid-A activity was the same as that described above. As seen from

Table 1. *Anti-lipid-A activity of immune sera obtained by immunization with various antigens*

Human red blood cells, coated with alkali-treated lipid A, were incubated with the antisera in the presence of complement. Bacteria were *S. minnesota* R595 and lipid A was from *S. minnesota* R345, except for serum 7 where lipid A from *E. coli* EH100 was used. The values represent reciprocal end titers. n.d. = not determined. The time of bleeding was on day 16 for intravenous immunization and on day 20 after the 2nd injection for subcutaneous immunization

Serum No.	Antigens used for immunization	Hemolysis test with sera obtained by	
		(a) intravenous immunization	(b) subcutaneous immunization (with incomplete Freund's adjuvant)
1a,b	Bacteria	n.d.	16
2a,b	Bacteria coated with lipid A	128	n.d.
3a,b	Acid-treated bacteria	128	128
4a,b	Acid-treated bacteria coated with lipid A	512	1024
5a,b	Freshly heat-killed bacteria	16	16
6a,b	Incomplete Freund's adjuvant	n.d.	< 4
7a,b	Acid-treated bacteria coated with lipid A	512	n.d.

Table 1 (serum 7), the *E. coli* lipid A was as immunogenic as the *Salmonella* lipid A preparation.

One immune serum (serum 4a of Table 1) was assayed with erythrocytes sensitized with lipid A from different sources. These lipid A preparations had been isolated from lipopolysaccharides (glycolipids) of the following strains which have been described previously [2,18,19]: *S. typhimurium* SR and TV161, *S. minnesota* R3, R5, and R595, and *E. coli* EH100. In all cases the hemolysis titers ranged from 1:250 to 1:512, showing that the different lipid A preparations were serologically indistinguishable.

*Specific Absorption and Desorption
of Lipid-A Antibodies*

The specificity of one anti-lipid-A antiserum (serum 4a of Table 1) was tested by absorption with formalinized erythrocytes sensitized with lipid A (from *S. minnesota* R345) as described in Materials and Methods.

As shown in Table 2, anti-lipid-A activity was completely absent from the absorbed serum, while in the control (treatment with non-sensitized erythrocytes) no change in titer was observed.

The absorbed anti-lipid-A antibodies could be dissociated by incubating the lipid-A-coated cells

Table 2. Absorption and isolation of lipid-A-specific antibodies from immune serum with lipid-A-sensitized erythrocytes
Reciprocal end titers are given

Serum preparations	Hemolysis test
Original antiserum (4a of Table 1)	512
Antiserum absorbed with: Formalinized red cells	512
Formalinized red cells coated with alkali-treated lipid A	4
Antibodies recovered after desorption from red cells	256

with glycine-HCl buffer at pH 2.2 [14]. This treatment led to a substantial recovery of anti-lipid-A activity as shown in Table 2.

Primary and Secondary Antibody Response to Lipid A

In this experiment each of two rabbits received two intravenous injections of acid-treated bacteria (*S. minnesota* R595) coated with lipid A (*S. minnesota* R345) (ratio 10:1, w/w), 100 µg on day 1, and 200 µg on day 15. At various intervals their serum was analyzed for anti-lipid-A activity. Fig. 1 shows the pattern of antibody response. Low levels of antibodies could be detected on day 4. After a steady rise, a maximum was reached on day 12. The booster injection on day 15 led to maximal titers on day 25, which then decreased.

Separation of Anti-Lipid-A Immunoglobulins from an Immune Serum

An anti-lipid-A immune serum (serum 4a of Table 1) was examined by column chromatography on Sephadex G-200 as described in Materials and Methods. Two main serologically-active protein peaks were resolved (Fig. 2), one corresponding to IgM (fractions 36 to 46) and the other corresponding to IgG (fractions 56 to 68). The fractions of each peak were pooled, dialyzed against Tris-glycine buffer (pH 7.6) and concentrated by negative pressure ultrafiltration each to half of the original volume. Both samples exhibited anti-lipid-A activity as determined in the passive hemolysis test. The titers were 1:180 for the IgM, and 1:250 for the IgG fraction (original titer: 1:512).

Serological Cross Reactions between Lipid A and Various S and R Form Lipopolysaccharides

The ability of anti-lipid-A antiserum (serum 4a of Table 1) to cross-react with S and R lipopolysaccharides from a variety of *Salmonella* and *E. coli* strains [2, 18, 19] was tested in hemolysis tests using

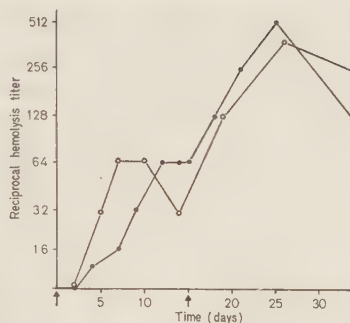


Fig. 1. Time course of antibody response to lipid A in two rabbits. Acid-treated bacteria (*S. minnesota* R595) coated with lipid A (*S. minnesota* R345) in a ratio of 10:1 (w/w) were injected intravenously on days 0 (100 µg) and 15 (200 µg) as indicated by arrows

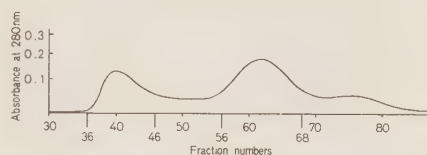


Fig. 2. Separation of anti-lipid A immunoglobulins on a Sephadex G-200 column. The serum used in this experiment is serum 4a of Table 1

red cells passively sensitized with these preparations (see also the following paper [31]).

As shown in Table 3, all S and R form lipopolysaccharides reacted with the anti-lipid-A serum (4a of Table 1). The highest titer (1:250) was obtained with the lipopolysaccharide (glycolipid) of the Re mutant of *S. minnesota* R595, which represents the most incomplete structure with the highest proportion of lipid A. Cross-reactions of various magnitude (titers between 1:64 and 1:4) were obtained with all other preparations. To demonstrate the specificity of these cross-reactions, the lipopolysaccharides were also tested with the same serum after absorption with lipid A. It was found that in all cases cross-reactions were now abolished. Furthermore, no interaction of the lipopolysaccharides with the pre-immunization serum was seen.

In a second series of experiments, a number of antibacterial antisera were tested for anti-lipid-A activity with erythrocytes sensitized with alkali-treated lipid A from *S. minnesota* R345. As seen from Table 3, eight out of thirteen antibacterial antisera exhibited anti-lipid-A activity with titers ranging from 1:4 to 1:128.

Table 3. Serological cross-reactions between various lipopolysaccharides and anti-lipid-A antiserum, and between lipid A and various anti-bacterial antisera

Human red blood cells were coated with lipopolysaccharides of S forms, alkali-treated lipopolysaccharides from R forms, and alkali-treated lipid A, respectively. Reciprocal end titers are given. n.d. = not determined

		Passive hemolysis test with	
Bacterial strains from which lipopolysaccharides and antisera were derived		Lipopolysaccharides and lipid A antiserum (4a of Table 1)	Lipid A and bacterial antisera
S forms	<i>S. typhimurium</i>	32	< 4
	<i>S. abortus equi</i>	16	< 4
	<i>S. typhi</i>	64	4
	<i>S. worthington</i>	32	32
	<i>S. minnesota</i>	64	< 4
	<i>S. godesberg</i>	32	< 4
	<i>S. milwaukee</i>	8	128
	<i>E. coli</i> O111	32	n.d.
R forms	<i>S. paratyphi</i> B T ₁	16	16
	<i>S. friedenaui</i> T ₁	4	< 4
	<i>S. typhimurium</i> TV161	32	n.d.
	<i>S. minnesota</i> R345	64	4
	<i>S. typhimurium</i> M	64	4
	<i>S. minnesota</i> R5	32	n.d.
	<i>S. minnesota</i> R7	16	n.d.
	<i>S. minnesota</i> R3	16	16
	<i>S. minnesota</i> R595	256	16
Lipid A		512	

Absence of Cross Reaction between Lipid A and Sheep Erythrocytes

Rabbit sera are known to contain antibodies which react serologically with antigens present on the surface of sheep red cells. The following experiments were performed to find out whether these antibodies cross-react with lipid A. Non-sensitized sheep red blood cells and lipid-A-sensitized human red blood cells were assayed comparatively with various sera in the hemolysis test. As seen from Table 4, normal rabbit serum lysed sheep cells up to a dilution of 1:360. Only a slightly higher titer, 1:640, was obtained with the anti-lipid-A antiserum (serum 4a of Table 1). Both titers were abolished (1:4) by absorption of the sera with sheep red blood cells, but no decrease was observed after absorption with lipid A. On the other hand, absorption of the anti-lipid-A immune serum with sheep red cells had no effect on the lipid-A titer. Furthermore, anti-sheep-cell antiserum, which lysed sheep cells to a high titer, did not cross-react with lipid A. All these results indicate that the anti-lipid-A antibodies, described in this paper, do not cross-react with antigens present on sheep red cells.

Table 4. Absence of cross reactivity between lipid A and sheep erythrocytes
Reciprocal end titers are given

Sera (rabbit)	Passive hemolysis test with	
	Sheep red cells	human red cells coated with alkali-treated lipid A
Normal serum	360	< 4
Anti-lipid-A antiserum: (4a of Table 1)	640	512
after absorption with sheep red cells	< 4	512
after absorption with lipid A-coated human red cells	640	< 4
Antiserum against sheep red cells*	> 1024	< 4

* Obtained from Behring Werke, Marburg.

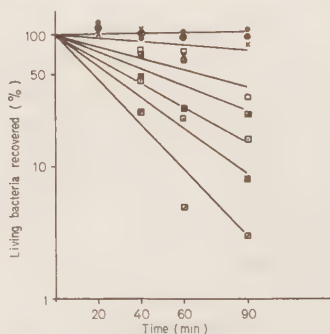


Fig. 3 Recovery from the mouse peritoneum of *E. coli* O111 bacteria non-opsonized (○) and opsonized with anti-lipid A immune serum. Opsonization was carried out with the antiserum (serum 4 of Table 1) in dilutions 1:500 (◻), 1:1000 (◼), 1:2000 (◻), 1:5000 (◼), 1:10000 (◻) and, as controls, with lipid A-adsorbed antiserum (1:500, ●), and with pre-immunization serum (1:1000, ×)

Opsonization of *E. coli* O111 Bacteria with Anti-Lipid-A Antiserum

The ability of lipid-A antiserum to promote intraperitoneal phagocytosis of bacteria in mice was tested with *E. coli* O111 bacteria which had been treated with various concentrations of normal and immune sera [16]. Constant numbers of bacteria were injected intraperitoneally into mice and the number of surviving bacteria was determined at various time intervals. The results are shown in Fig. 3.

In the animals which had received either bacteria alone or bacteria incubated with normal serum, no significant reduction in the number of viable bacteria took place within 90 min. In contrast, 97 and 90%

killing was obtained when the bacteria had been treated with the anti-lipid-A serum at dilutions of 1:500 or 1:1000, respectively. At higher dilutions, the rate of killing decreased, but even treatment of the bacteria with antiserum diluted 1:10000 resulted in 70% killing. In order to show that phagocytosis was being promoted by anti-lipid-A antibodies, the antiserum was absorbed with lipid-A-coated erythrocytes. As seen in Fig. 3 this absorbed serum no longer induced phagocytosis.

DISCUSSION

Immunochemical studies on the lipopolysaccharides (O antigens) of gram-negative bacteria have revealed that discrete structures in the O-specific polysaccharide chains function as the serological determinants against which the O antibodies are directed [1,20]. In incomplete lipopolysaccharides of R mutants, oligosaccharides of the basal core represent the determinant regions [2]. In addition to these specific O and R antigens, a number of antigens present on the enterobacterial cell have been described, which exhibit, under certain conditions, common specificities, thus causing cross-reactions between species [21-23]. The best known example is the common antigen of Kunin [21,24], which is present on the surface of all Enterobacteriaceae. This antigen, however, is detectable only with antisera obtained with a limited number of species, which carry the common antigen in an immunogenic form. In most strains, its immunogenicity is not expressed due to the presence of lipopolysaccharide [25].

A quite similar situation seems to exist with regard to lipid A, which is a common component of all lipopolysaccharides, and structurally identical or similar for Enterobacteriaceae [26]. Thus, the formation of antibodies against this common structure of lipopolysaccharides might be expected, but such antibodies have never been described. In fact, the results presented in this paper indicate that the formation of anti-lipid-A antibodies is suppressed to a large extent when immunization is performed with lipopolysaccharides or bacterial cells, in which lipid A carries long polysaccharide chains as in S forms or oligosaccharide units as in R mutants.

On the other hand, the results obtained in this investigation show clearly that lipid A, when presented to the animal (rabbit) in a suitable form, can act as an immunogen. Immunization with intact *S. minnesota* R595 bacteria, containing on their cell surface the glycolipid 2-keto-3-deoxyoctonate-lipid A resulted in low levels of anti-lipid-A activity. Improved titers were obtained by immunization with the same bacteria from which 2-keto-3-deoxyoctonate had been cleaved off by mild acid hydrolysis. Such hydrolyzed bacteria contain free lipid A in their outer cell membrane. When these hydrolyzed bacteria were

coated with additional free lipid A, isolated from a lipopolysaccharide by mild acid hydrolysis, and used for immunization, the highest anti-lipid-A titers so far were obtained and the antibody formation shows a time course which resembles that of antibody formation against the polysaccharide structure of lipopolysaccharides [27]. In general, the antibody response to subcutaneous immunization with Freund's adjuvant was slightly higher than to the intravenous immunization scheme. The results show that lipid A, when sufficiently exposed on the bacterial cell, stimulates the formation of specific antibodies in the animal.

For the determination of anti-lipid-A activity in such antisera, the passive hemolysis test proved to be sensitive and convenient (another specific assay for anti-lipid-A activity involving liposomal model membranes is described in the following paper [31]). Human-blood-group B Rh⁺ cells, which show no cross reaction with normal rabbit serum or with anti-lipid-A antisera, could be coated readily with lipid A solubilized either with triethylamine [11] or by alkali-treatment [17]. With this test, antibodies against lipid A were detected in the IgM and IgG fractions when the immune serum was resolved on Sephadex G-200. Lipid-A-sensitized erythrocytes were also used for the absorption of lipid-A antibodies from immune sera and their reisolation. The lipid-A specificity of the immune sera could thus be demonstrated.

The finding that lipid A preparations, obtained from one *E. coli* and a number of *Salmonella* lipopolysaccharides, exhibited strong serological cross reactions with different lipid-A antisera was to be expected. However, regarding a possible biological role of anti-lipid-A antibodies, the question arose, if these antibodies would also cross-react with lipopolysaccharides of S and R forms, which contain lipid A in a cryptic position as a result of the presence of polysaccharide chains attached to it. The results of Table 3 show that all lipopolysaccharides tested cross-react with the lipid-A antiserum, some to a high degree. On the other hand, not all anti-bacterial antisera (prepared routinely by immunization of rabbits with heat-killed bacteria) exhibited anti-lipid-A activity. This means that although lipid A on the bacteria is not always sufficiently exposed for induction of antibodies, anti-lipid-A antibodies are capable of reacting with lipopolysaccharides, even with those of S forms. It is well known that antibodies directed against surface structures of the bacterial cell play an important role in defense mechanisms to infection [16]. The finding that anti-lipid-A antibodies are capable of reacting with lipopolysaccharides indicated that they might function as opsonins and promote phagocytosis of bacteria. To investigate this possibility, one system has been studied so far. *E. coli* O111 bacteria were treated with lipid-A antiserum and their fate in the mouse peritoneum was followed. It was found that anti-lipid-A antibodies were effective

at high dilutions in sensitizing these bacteria for subsequent phagocytosis.

Thus, anti-lipid-A antibodies are capable of reacting with lipopolysaccharides present on the cell surface of S form bacteria. How far this is true for Enterobacteriaceae in general is a very important question. Since all enterobacterial cell walls contain lipid A, highly specific lipid-A antiserum may be found to be reactive universally with a great number of otherwise serologically distinct species. Studies are under way to find out whether anti-lipid-A antibodies can afford protection to enterobacterial infections. In this connection it should be emphasized that lipid A represents the endotoxic principle of lipopolysaccharides [32]. It is therefore possible that antibodies which are able to react specifically with lipid A might also exhibit anti-endotoxic activity.

At present, it is not known which structures in lipid A exert immunodominant functions. Lipid A is built up of glucosaminyl- β -1,6-glucosamine disaccharides, substituted in 1 and 4' positions by phosphate groups which may serve as bridges to adjacent glucosamine disaccharides [3]. The hydroxyl groups (with the exception of that at 3') are substituted by long chain fatty acids (lauric, myristic, palmitic and β -hydroxymyristic acid), and the amino groups by β -hydroxymyristic acid [28]. Amino acids (about 0.1%) are also present [7]. It should be mentioned that alkali-treated lipid A, from which a large proportion of ester fatty acids has been removed, reacts equally well as the intact lipid A with anti-lipid-A antibodies.

Recently, Rank *et al.* [29,30] have described anti-lipid-A antibodies, which were obtained by immunization of mice with lipopolysaccharides and lipid A, and which were identified *in vitro* (Jerne technique) by their cross reaction with sheep red cells. The anti-lipid-A antisera described in this paper did not show any cross reaction with surface antigens of sheep red cells, and are, therefore, certainly distinct from those characterized by Rank *et al.* [29,30]. They are also serologically distinct from anti-common-antigen antibodies of the Kunin type [21,24], since these do not interact with lipid-A-coated red cells [33].

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VIII

Detection and Specificity of Lipid A Antibodies Using Liposomes Sensitized with Lipid A and Bacterial Lipopolysaccharides

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Previous reports have shown that liposomal model membranes are appropriate objects for investigating the molecular basis of complement-dependent membrane damage. The present study describes a different application for liposomes: their usefulness in screening sera suspected of containing antibodies against potential lipid antigens. Liposomes, actively sensitized with untreated or alkali-treated lipid A derived from *Salmonella minnesota*, were found to release trapped glucose marker when incubated with a source of unheated complement and the anti-lipid A serum whose preparation is described in the preceding paper [1]. In the presence of complement, the anti-lipid A serum could also promote loss of glucose from liposomes which had been actively sensitized with various untreated and alkali-treated S or R form *S. minnesota* lipopolysaccharides. In contrast, several different anti-R-form sera had little effect on liposomes sensitized with either untreated or alkali-treated lipid A. These results are in agreement with observations on the immunological specificity of lipid-A antibodies using complement-dependent hemolysis of passively sensitized human erythrocytes as test system. Alkali-treated, but not untreated, lipid A could also be employed for passive sensitization of liposomes; in this regard, lipid A behaves similarly to S and R form lipopolysaccharides.

In the preceding paper, Galanos *et al.* [1] have demonstrated that lipid A derived from gram-negative bacteria may function as an immunogen giving rise to specific antibodies in rabbits. In this report, we wish to record some additional evidence which supports these observations.

For this purpose, use was made of liposomes which release trapped glucose marker when incubated with an appropriate antiserum and complement source. These model membranes have been shown previously to be appropriate objects for investigating the molecular basis of complement-dependent lysis (*cf.* [2–7] and additional references cited therein). The current investigation describes a different application for such liposomes: their usefulness in screening sera suspected of containing antibodies against potential lipid antigens.

In the earlier studies, sensitization of liposomes was accomplished with amphipathic antigens whose immunogenicity and immunochemical properties had been well established. These were ceramide homologs (such as galactocerebroside, globoside I, and Forssman antigen) and bacterial lipopolysaccharides (untreated or alkali-treated S and R forms derived from *Salmonella minnesota*). The present experiments demonstrate that liposomes prepared with lipid A constitute a sensitive system for the identification and characterization of lipid A antibodies. They are

virtually identical in their response to antibody and complement with those sensitized with the lipopolysaccharides [7].

MATERIALS AND METHODS

Lipopolysaccharides and Lipid A

The S and R form lipopolysaccharide preparations (untreated and alkali-treated) were the same as those employed in a previous study [7]. Free lipid A was isolated by acetic acid hydrolysis of *S. minnesota* R595 lipopolysaccharide as described in the preceding paper [1]; the latter should also be consulted for the procedures involved in alkali treatment of the isolated lipid A.

To prepare stock solutions of the untreated and alkali-treated lipopolysaccharides, or alkali-treated lipid A, suspensions (5 mg/ml distilled water) were sonicated for 30–60 sec using a Biosonik (Bronwill Scientific, Rochester, N.Y.) with a microprobe operated at a power setting of about 10. As noted earlier [7], these solutions displayed only very slight opalescence and were stable at room temperature. However, in the case of untreated lipid A, it was necessary to perform the sonication at a higher probe intensity (about 60) and, because reaggregation quickly occurred, appropriate aliquots were immediately dispensed into the flasks used for liposome preparation (see below).

Antisera

Rabbit antisera to the various S and R form lipopolysaccharides were also the same as those employed previously [7]. Antiserum to lipid A was prepared by the intravenous immunization scheme described in the preceding paper using as antigen hydrolyzed bacteria (*S. minnesota* R595) that had been coated with mR345 lipid A [1]. All antisera were decomplemented by heating for 30 min at 56 °C.

Liposome Preparation

Liposomes sensitized with either untreated or alkali-treated lipid A were generated by the procedures developed for the preparation of liposomes with various lipopolysaccharides; complete details are given in [7]. Briefly, this involved deposition of a lipid film (composition: 2 μ moles of lecithin or sphingomyelin, 1.5 μ moles of cholesterol, and 0.22 μ moles of dicetyl phosphate) on the walls of a 10 ml conical flask. Active sensitization of the liposomes was accomplished by dispersing the dried lipids with a Vortex mixer in 0.2 ml of a solution containing 300 mM glucose and the desired amount of antigen. The liposome preparation was then dialyzed against an isotonic salt mixture [3] to remove most of the untrapped glucose. Passive sensitization was performed by dispersing the lipids in 0.2 ml of a solution containing only 300 mM glucose, i.e. antigen was not present at the time the liposomes were generated. Dialyzed liposomes were subsequently incubated with appropriate quantities of untreated or alkali-treated lipid A in the assay cuvettes (see legend to Fig. 3 for additional details).

Glucose Release Assay

The identical spectrophotometric method used for following loss of marker from lipopolysaccharide-sensitized liposomes [7] was employed in the present investigation. A full description of this assay has been published previously [3] and therefore only a summary of the essential steps is presented here; this will be subsequently referred to as the standard assay procedure.

500 μ l of an assay reagent (containing ATP, NADP⁺, Mg²⁺, Ca²⁺, hexokinase, and glucose-6-phosphate dehydrogenase in Tris-saline buffer; see [3] for exact composition) were added to cuvettes with a 10 mm light path, followed by 360 μ l of veronal buffer, 13 μ l of antiserum, and 113 μ l of guinea-pig serum. The absorbance at 340 nm was measured and then 5 μ l of liposome preparation was added; the absorbance was again determined after 30 min incubation at room temperature. The initial value was subtracted from the final value to provide a measure of the amount of glucose released plus the contributions to the absorbance of any untrapped glucose in, and light scatter by, the liposome preparation. To correct

for the latter variables, the above procedure was repeated in assay cuvettes which contained 126 μ l of veronal buffer instead of the antiserum and guinea pig serum; the absorbance difference was obtained from values determined before and approximately 5 min after liposome addition.

As in the earlier studies [2–7], results are expressed as the percentage of trapped glucose released in the presence of antiserum and complement. The trapped glucose (equivalent to the total minus the untrapped marker in the liposome preparations) was determined as specified in [3]. In this regard, it should be noted that the latter contains a typographical error in the description of the chloroform-Triton procedure by which total glucose was measured; a correction has appeared [8].

RESULTS AND DISCUSSION

Fig. 1 and 2 illustrate that liposomes actively sensitized with either untreated or alkali-treated lipid A release trapped glucose marker when incubated with anti-lipid A serum and guinea-pig serum as source of complement. The extent of marker loss was dependent on the amount of antigen initially present during preparation of the liposomes. This is analogous to the response of liposomes sensitized with various ceramide homologs [3, 6] or *Salmonella* lipopolysaccharides [7].

Experiments with the lipopolysaccharides have shown that alkali treatment of either S or R forms, which results in the removal of approximately 50% of the esterified long chain fatty acids from the lipid A region, produces a more effective antigen for liposomal sensitization [7]. This was indicated by a marked reduction in the amount of alkali-treated material, compared to untreated lipopolysaccharide, required for half-maximal sensitization, i.e. half-maximal glucose release in the presence of excess antiserum and complement source. It was presumed that this reflected a change in the solubility characteristics of the antigens (perhaps an increase in critical micelle concentration) which favored formation of an antigen species more readily incorporated into or absorbed onto the lipid bilayers. Further support for the above assumption was provided by the observation that very small amounts of lipid A were needed for active liposomal sensitization after the material had been rendered more water-soluble by alkaline hydrolysis (compare abscissae of Fig. 1 and 2).

Fig. 1 and 2 also describe another phenomenon which we have encountered before: liposomes prepared with lecithin generally lose a greater percentage of their trapped marker than those made with sphingomyelin. This was particularly pronounced in the present investigation when untreated lipid A was employed as the sensitizing antigen (Fig. 1); here the difference in the percentage glucose released from lecithin *versus* sphingomyelin liposomes approached

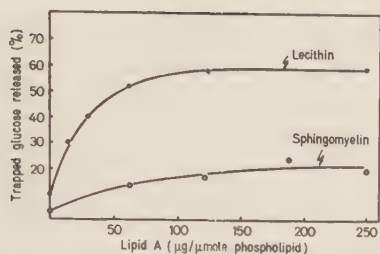


Fig. 1. Effect of untreated lipid A on the immune sensitivity of liposomes prepared with lecithin or sphingomyelin. Liposomes were prepared from lipid mixtures containing either lecithin or sphingomyelin in the presence of varying amounts of untreated lipid A as indicated on the abscissa. Glucose release was determined by the standard assay procedure, and is expressed as the percentage released in 30 min. ●, lecithin; ○, sphingomyelin

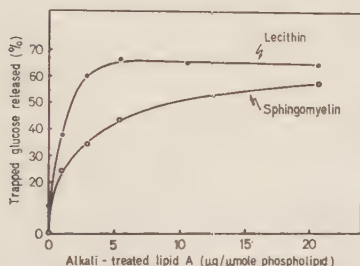


Fig. 2. Effect of alkali-treated lipid A on the immune sensitivity of liposomes prepared with lecithin or sphingomyelin. Liposomes were prepared from lipid mixtures containing either lecithin or sphingomyelin in the presence of varying amounts of alkali-treated lipid A as indicated on the abscissa. Glucose release was determined by the standard assay procedure, and is expressed as the percentage released in 30 min. ●, lecithin; ○, sphingomyelin

40%. In earlier studies using liposomes sensitized with ceramides [6] or lipopolysaccharides [7], the difference was significantly less (5 to 20%) but was sufficiently reproducible to warrant speculation that phospholipid composition may be an important factor in determining the sensitivity of membranes to immune damage [7].

Recent experiments with liposomes prepared from radioactive phospholipids [4], or synthetic phospholipid and phospholipid analogs [5], suggest that complement-dependent membrane damage does not occur by the direct action of phospholipases A, B, C, or D. Thus, it does not appear that the difference in liposomal response reflects the abilities of lecithin and

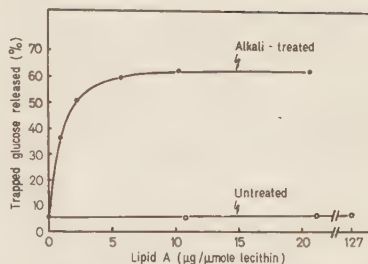


Fig. 3. Passive sensitization of liposomes prepared with lecithin by untreated and alkali-treated lipid A. Liposomes were prepared from a lipid mixture containing lecithin in the absence of any lipid A. Glucose release was determined by a modification of the standard assay procedure in which the liposomes were preincubated in cuvettes containing sufficient amounts of a solution of either untreated or alkali-treated lipid A to give the final ratios indicated on the abscissa. After 10 min at room temperature, the reaction was initiated by the addition of 113 μl of guinea-pig serum followed by 13 μl of anti-lipid-A serum, glucose release is expressed as percentage released in 30 min. ●, alkali-treated lipid A; ○, untreated lipid A

sphingomyelin to serve as substrates for these enzymatic activities which conceivably could be generated as a consequence of activation of the complement sequence. As discussed elsewhere [6], one of the factors which may contribute to a smaller percentage loss of glucose is a greater intrinsic stability of sphingomyelin-cholesterol, as opposed to lecithin-cholesterol, bilayers. In this regard, it should be noted that, even when antigen is omitted, more marker is lost ("non-immune" glucose release) under conditions of assay from liposomes made with lecithin than from those prepared with sphingomyelin (Fig. 1 and 2). In view of their greater sensitivity, only lecithin liposomes were employed in subsequent experiments concerned with antibody specificity, complement requirement, and passive sensitization.

Fig. 3 shows that alkali-treated, but not untreated, lipid A could passively sensitize liposomes to the action of antibody and complement. Up to now, alkali-treated S and R form lipopolysaccharides were unique in that, of all the antigens used to prepare immunologically responsive liposomes, only these have been effective in the passive sensitization procedure [7]. The present experiment therefore indicates that this capacity can be attributed to the removal by alkaline hydrolysis of fatty acids from the lipid A portion of these macromolecular antigens rather than to some hitherto undetected alteration in the polysaccharide (O antigen or core) regions.

Fig. 4 and 5 illustrate that the extent of glucose release from liposomes sensitized with either untreated or alkali-treated lipid A is dependent on the amounts

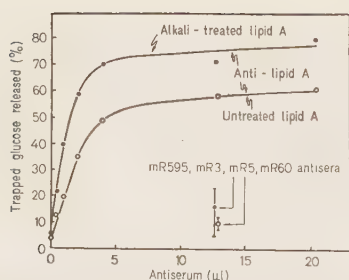


Fig. 4. Effect of antiserum concentration on liposomes prepared with lecithin. Liposomes were prepared from a lipid mixture containing lecithin in the presence of sufficient untreated or alkali-treated lipid A to give 125 $\mu\text{g}/\mu\text{mole}$ lecithin or 21 $\mu\text{g}/\mu\text{mole}$ lecithin, respectively. The standard assay procedure for determination of glucose release was modified by the addition of varying amounts of anti-lipid A serum as indicated on the abscissa. It is expressed as percentage released in 30 min. The bars indicate the average and range of the percentage of trapped glucose released from the liposomes when 13 μl of anti-mR595, anti-mR3, anti-mR5, or anti-mR60 sera were substituted for the anti-lipid A serum in the standard assay procedure

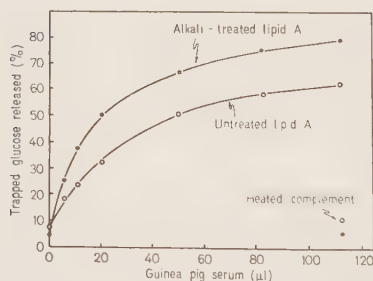


Fig. 5. Effect of guinea-pig serum concentration on liposomes prepared with lecithin. Liposomes were prepared from a lipid mixture containing lecithin in the presence of sufficient untreated or alkali-treated lipid A to give the ratios 125 $\mu\text{g}/\mu\text{mole}$ lecithin or 21 $\mu\text{g}/\mu\text{mole}$ lecithin, respectively. The standard assay procedure for determination of glucose release was modified by the addition of varying amounts of guinea pig serum as indicated on the abscissa. It is expressed as percentage released in 30 min. Heated complement refers to guinea-pig serum incubated at 56 $^{\circ}\text{C}$ for 30 min

of anti-lipid A serum and guinea-pig serum present during assay; the latter does not function after heating for 30 min at 56 $^{\circ}\text{C}$ to destroy hemolytic complement activity (Fig. 5). Although these experiments were performed with liposomes that had been actively sensitized, it should be noted that unheated guinea-pig serum was also required to demonstrate an effect

Table 1. Effect of anti-lipid A and homologous antiserum on lecithin liposomes prepared with lipid A or different lipopoly-saccharides

Liposomes were prepared from lipid mixtures containing lecithin in the presence of lipid A or the various lipopoly-saccharides specified in the far left column. When untreated lipopoly-saccharide or lipid A was employed as antigen, the final ratio was 125 $\mu\text{g}/\mu\text{mole}$ lecithin; in the case of the alkali-treated lipopoly-saccharides, the final ratio was 21 $\mu\text{g}/\mu\text{mole}$ lecithin. Glucose release was determined by the standard assay procedure in the presence of either 13 μl anti-lipid-A serum or 13 μl of homologous antiserum; the latter values were obtained from experiments described in [7]

Antigen	Trapped glucose released by:	
	Anti-lipid-A serum	Homologous antiserum
None	11.4	8.4
Lipid A	45.5	—
Lipopoly-saccharide:		(average)
mR595	76.6	77.5
Alkali-treated mR595	74.5	76.2
mR3	53.4	n.d.
mR5	45.0	63.0
Alkali-treated mR5	65.2	87.5
mR60	23.0	63.0
S	23.4	22.4
Alkali-treated S	51.3	62.5

of anti-lipid A serum on liposomes that were passively sensitized with alkali-treated lipid A (results not shown).

As regards the immunological specificity of liposomal response, Fig. 4 demonstrates that various anti-R form sera have very low levels, if any, of anti-lipid A activity. These observations are in complete accordance with results obtained by Galanos *et al.* [1] who have employed complement-dependent hemolysis of passively sensitized human erythrocytes as the test system. With this method, they were also able to show that anti-lipid A serum could cross-react with different R and S form lipopoly-saccharides. The experiments recorded in Table 1 support this conclusion and indicate that, in the presence of excess native guinea-pig serum, anti-lipid A serum can release nearly the same amount of glucose as the homologous antisera from liposomes sensitized with various untreated or alkali-treated R and S form lipopoly-saccharides. The agreement between the different assay systems is particularly gratifying because, as noted in the Introduction, one of the primary goals of this study was to determine whether the response of liposomes could be used to detect and characterize certain antibodies capable of reacting with suspected amphipathic antigens. The potential of this method for the detection of antibodies against a variety of pharmacological agents (*e.g.* drugs, hormones) is currently under investigation.

As regards the biological significance of these observations, it is important to emphasize that both an appropriate antibody source and complement are necessary to obtain glucose release from liposomes sensitized with lipid A and, as shown previously [7], S or R form lipopolysaccharides. This dual requirement in the case of lipopolysaccharides was somewhat surprising because extensive studies have indicated that these materials can interact with the complement system in the absence of exogenous antibodies as revealed, for example, by formation of characteristic lesions on the lipopolysaccharide surface, consumption of late-acting complement components, and generation of chemotactic factors (see [9] for review). Although the participation of very low levels of endogenous antibodies in these phenomena still remains a possibility, recent experiments by Galanos *et al.* [10] provide at least a partial explanation for this discrepancy. They have shown: that the ability to interact directly with complement (*i.e.* in the absence of added antibody) is not a general property of all lipopolysaccharides; that lipopolysaccharides which fix complement are no longer able to do so when rendered insoluble in the presence of Mg^{2+} or Ca^{2+} ions; and also that lipid A is the active moiety in these reactions because the latter *per se* can interact with complement but only if solubilized by a suitable carrier. The results obtained with lipid-A-sensitized liposomes (*i.e.* antiserum requirement for glucose release) thus support the previous suggestion that the region of the lipopolysaccharide molecule which is responsible for direct interaction may be "buried" upon incorporation into liposomal bilayers [7]. This suggestion is also consistent with the model proposed by Rothfield and Romeo for the orientation of R form lipopolysaccharides in lipid monolayers [11]. On the basis of these findings, it would appear advisable to determine whether lipid A portions are sufficiently "exposed" in the bacterial cell-wall/membrane struc-

ture in order to evaluate the biological importance of direct interaction with the complement system (or pro-activators of the latter) that has been observed with some isolated lipopolysaccharides.

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